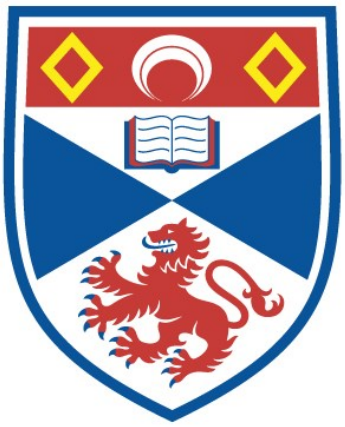
STUDIES ON THE NADH-NITRATE REDUCTASE FROM BARLEY

Ian Stewart Small

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1980

STUDIES ON THE NADH-NITRATE REDUCTASE FROM BARLEY

by

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A thesis submitted to the University of St. Andrews in application for the degree of Doctor of Philosophy

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January 1980

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry and Microbiology of the University of St. Andrews under the direction of Dr. J.L. Wray.

CERTIFICATE

I hereby declare that Ian Stewart Small has spent nine terms in research work under my direction and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews), and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1972 and graduated with the degree of Bachelor of Science, Upper Second Class Honours in Biochemistry in July 1976.

In October 1976, I matriculated as a research student at the University of St. Andrews.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. J.L. Wray for his encouragement, advice and criticism throughout this work; the Science Research Council for the award of a studentship; Mr. D. Kirk for his technical assistance and Mrs. L. Creswell for the typing.

SUMMARY

The structure and properties of barley nitrate reductase (NADH : nitrate oxidoreductase, E.C. 1.6.6.1.) have been analysed. A protocol has been established for the purification of barley nitrate reductase, involving $(NH_4)_2SO_4$ fractionation, gel filtration through Biogel A1.5 m and affinity chromatography through Blue Dextran-Sepharose. This protocol should allow the preparation of homogeneous enzyme at higher yields than have hitherto been obtained, although this goal was not achieved in the work reported here.

Barley nitrate reductase has been shown to have a calculated molecular weight of 203 000, based on experimentally determined values for its sedimentation coefficient (7.7 S) and Stokes radius (6.4 nm). The enzyme is highly asymmetrical with a frictional ratio of 1.65 and an axial ratio of 11:1. It can catalyse the reduction of nitrate using either NADH (the physiological electron donor), FMNH or reduced methyl viologen as electron donor and can also catalyse an NADH-dependent reduction of cytochrome c.

Barley nitrate reductase is shown to break down to give rise to NADH-cytochrome c reductase species possessing molecular weights of 40 000, 61 000, and 163 000 and the levels of these species are shown to be much higher in extracts from plants older than 90 hours than in extracts

from 90-hour old plants. A purification procedure for the 40 000 molecular weight NADH-cytochrome c reductase species has been established and involves Blue Dextran-Sepharose affinity chromatography.

Data is presented which characterises a ferrocyanide-activated NADH-cytochrome c reductase species in extracts from barley plants and shows it to be a globular protein with a molecular weight of 45 000. Evidence is presented which suggests that this species is not related to nitrate reductase.

The ability of artificial electron acceptors to accept electrons from nitrate reductase in place of cytochrome c has been examined. It is concluded that although DCPIP, ferricyanide and nitroblue-tetrazolium are capable of acting as substrates for the reaction, they do so much less efficiently than cytochrome c and are much less specific than cytochrome c.

The thermal stability of all the associated activities of barley nitrate reductase at 45° has been examined. Whereas the half-lives of both NADH-dependent activities were short (3-5 minutes) those of FMNH- and reduced methyl viologen nitrate reductase activities were much longer (23.5 minutes and 35 minutes respectively). Thermal inactivation of FMNH-nitrate reductase-activity was shown to be biphasic and evidence is presented which suggests that FMNH may donate electrons at two separate sites on

barley nitrate reductase.

Attempts were made to reconstitute nitrate reductase activity *in vitro* from the isolated 40 000 molecular weight NADH-cytochrome c reductase species and MCC derived from acid-treated barley nitrate reductase. These were unsuccessful.

In the General Discussion, the evidence available regarding the structure of the nitrate reductases from fungal, algal and higher plant sources is reviewed and analysed. A model for the structure of higher plant nitrate reductase is presented which accounts for the data reported here and in the literature. A mechanism for the turnover of nitrate reductase *in vivo* is also proposed as is the probable route of genetic evolution of the nitrate reductases.

ABBREVIATIONS

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cyt	cytochrome
DCPIP	2,6-dichlorophenol-indophenol
EDTA	Ethylenediamine tetraacetic Acid
FAD	Flavin Adenine Dinucleotide
FMN(H)	Flavin Mononucleotide-oxidised (reduced)
Methyl Viologen	1,1'-Dimethyl-4,4'-bipyridilium dichloride
MCC	Molybdenum containing Component
NAD^+	Nicotinamide Adenine Dinucleotide - oxidised form
NADH	Nicotinamide Adenine Dinucleotide - reduced form
NBT, Nitroblue tetrazolium	2,2'-Di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4-diphenylene)ditetrazolium chloride
pCMB	p-chloromercuribenzoate
RuBP	Ribulose 1,5-biphosphate
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic Acid
TEMED	N,N,N',N'-tetramethylethylene-diamine
Tris	Tris-hydroxymethylmethylamine.

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INTRODUCTION

1. Nitrogen Assimilation in Plants

Some plants, notably the legumes, e.g. soyabean, pea and peanut, are capable of forming a symbiotic relationship with certain *Rhizobia* species of bacteria to form root nodules in which atmospheric N_2 may be fixed directly into ammonium (Brill, 1977). Most plants, however, depend entirely for growth on fixed nitrogen absorbed from the soil mainly as nitrate but also as ammonium although this latter source is rapidly converted to nitrate by *Nitrosomonas* and *Nitrobacter* species in the soil (see Fowden, 1979). Nitrate uptake is thought to be triggered in response to nitrate by the induction of a specific permease (Jackson, Flesher and Hageman, 1973) and there is evidence that both nitrate uptake and reduction are coordinately induced (Butz and Jackson, 1977).

Nitrate enters the plant through the root plasma membrane and may then be either (a) reduced in the root to form nitrite, ammonium and eventually α-amino nitrogen, (b) transferred to the xylem for transport to the aerial parts of the plant, or (c) stored in the leaf vacuole for later use (Oaks, 1979). In cotton plants it has been shown (Radin, 1977) that the bulk of the nitrate taken up by the root is transported to the leaves and the amount reduced in the roots is only barely sufficient to meet their synthetic requirements.

Nitrate transported to the shoots may undergo either

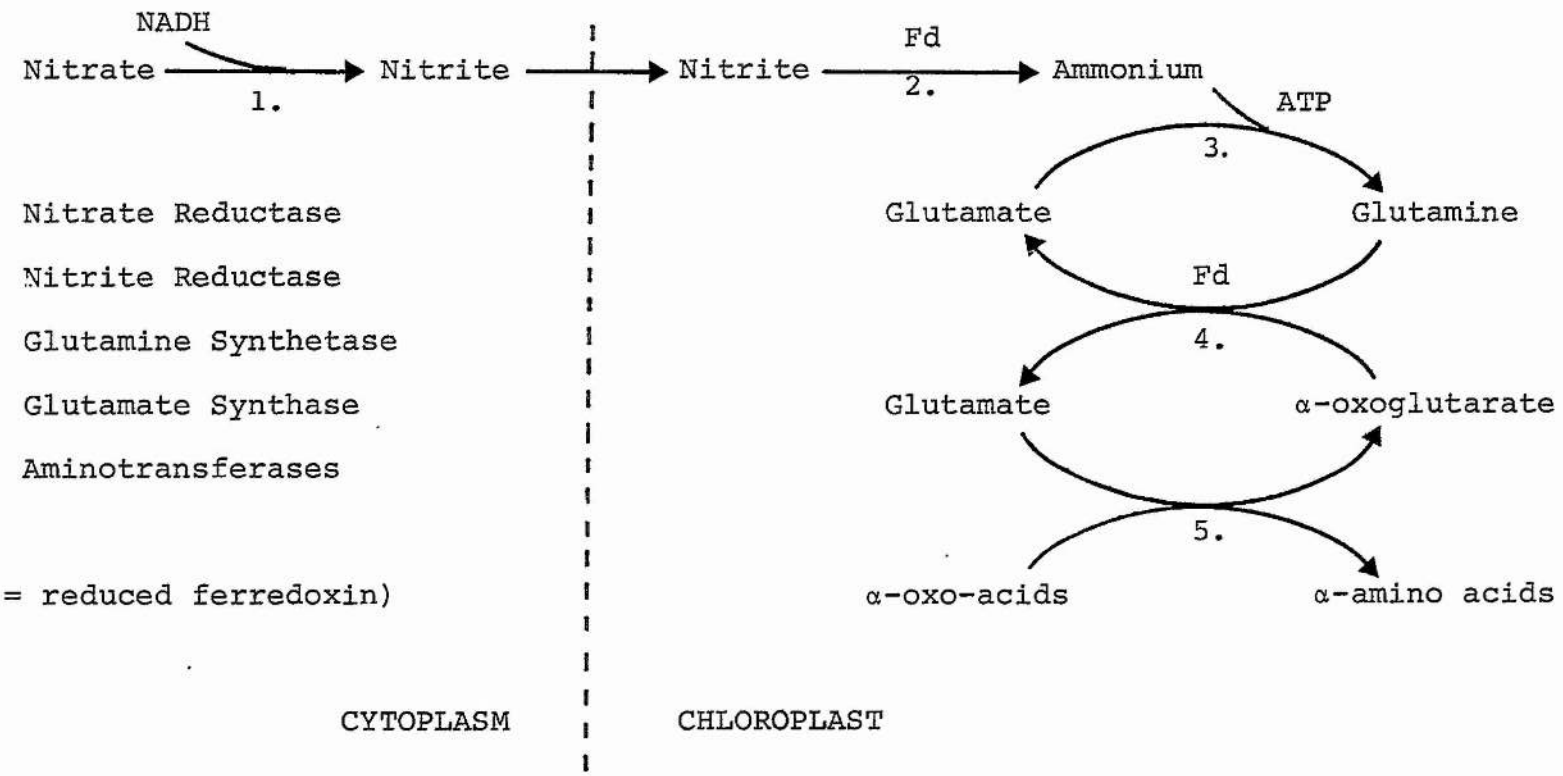
storage, or alternatively reduction by nitrate reductase (Ferrari, Yoder and Filner, 1973; Pate, 1973) and there is evidence (Shaner and Boyer, 1976) that the level of nitrate reductase activity in the shoots is controlled by the rate of nitrate flux to the shoots, through the xylem, rather than by the total nitrate content of the shoots. Nitrate reductase activity in the shoots results in the formation of nitrite which enters the chloroplasts and is further reduced to ammonium by nitrite reductase, to which photosynthetically reduced ferredoxin donates electrons (Vega and Kamin, 1977).

The resultant ammonium is further metabolised within the chloroplast and is assimilated into glutamine by glutamine synthetase. This glutamine then reacts with α-oxoglutarate in a reaction catalysed by glutamate synthase (also called GOGAT, or L-glutamate, $NADP^+$ oxidoreductase (transaminating)) to produce two molecules of glutamate (Miflin and Lea, 1976; 1977). The glutamate formed in these reactions can then be used in the synthesis of the other protein amino acids, as well as for the synthesis of purines and pyrimidines. An outline of this nitrate assimilation pathway is presented in Scheme 1.

The key importance of nitrate reductase in plant nitrogen metabolism stems from the fact that nitrate is the major source of nitrogen for the plant. Also, as it is both the first, and the rate-limiting, step of the pathway (Beevers and Hageman, 1969) it follows that regulation

SCHEME 1

PATHWAY OF NITROGEN ASSIMILATION IN HIGHER PLANTS



of nitrate reductase automatically regulates the pathway as a whole. Hence, a great deal of effort has been made by many workers to understand both the structure and regulation of nitrate reductase in the hope that this knowledge may one day allow manipulation of the pathway to man's advantage. It is because of this that nitrate reductase is, arguably, the most studied higher plant enzyme.

2. Dissimilatory (Respiratory) Nitrate Reductases

Higher plant nitrate reductases, and those from fungal and algal sources to be described in this thesis, are all assimilatory enzymes in that they are involved in the assimilation of nitrate into amino acids and proteins. However, another class of nitrate reductases, known as the dissimilatory or respiratory nitrate reductases, occur in many bacteria grown under anaerobic conditions in the presence of nitrate. Under these conditions, nitrate acts as a terminal electron acceptor in place of oxygen allowing the respiratory generation of ATP.

E. coli nitrate reductase is typical of these enzymes in that it is located in the cytoplasmic membrane and consists of two types of polypeptide chain, having molecular weights of 155 000 and 65 000 respectively. The enzyme is often found in association with a cyt $b_{556}^{NO_3}$, which may be a component of the enzyme (MacGregor, 1975). The

involvement of Mo, non-haem iron and labile sulphide has been demonstrated in *E. coli* nitrate reductase and further details can be found in two recent reviews (Stouthamer, 1976; Ruiz-Herrera, 1978).

3. Characterisation of the Assimilatory Nitrate Reductases

(a) Molybdenum

The involvement of Mo in nitrate reduction was first suggested by the work of Steinberg (1937) who demonstrated that Mo-deficient cultures of the fungus *Aspergillus niger* were unable to grow when nitrate was supplied as sole nitrogen source. Similar results were obtained with Mo-deficient spinach plants (Hewitt, 1951) and it was shown that the plants were able to grow on NH_4^+ as nitrogen source. The Mo-requiring step was identified by Spencer and Wood (1954) to be the reduction of NO_3^- to NO_2^-. Further support for this conclusion was obtained (Evans and Hall, 1955; Anacker and Stoy, 1958) showing that the Mo co-purified with nitrate reductase activity.

Work with *Neurospora crassa* nitrate reductase (Nicholas and Nason, 1954a, b, c) had meanwhile shown that Mo was not only a component of the enzyme but was also involved in the transfer of electrons from NADPH to nitrate. The metal-binding agents cyanide and azide were shown to prevent the reduction of nitrate.

Further support for the role of Mo in nitrate reductase has come from the use of tungsten (W), an analogue of Mo. Hewitt (1951) and Agarwala (1952) demonstrated that plants grown in the presence of W were unable to grow when nitrate was supplied as sole nitrogen source and that addition of Mo relieved this inhibition. Heimer, Wray and Filner (1969) showed that growth in the presence of W prevented the formation of an active nitrate reductase and Wray and Filner (1970) subsequently showed that in the presence of W an intact nitrate reductase molecule was formed but was inactive.

Subsequently, Notton and Hewitt (1971a) have demonstrated the incorporation of radioactive Mo into a band which also possessed nitrate reductase activity following polyacrylamide gel electrophoresis of a partially purified sample. It was not found possible to reactivate nitrate reductase *in vitro* from a W-grown plant by the addition of molybdate (Notton and Hewitt, 1971c) indicating that the two metals are not interchangeable *in vitro*.

(b) Flavin

The involvement of a flavin component in nitrate reduction was first suggested by Evans and Hall (1953) who demonstrated that the reduction of nitrate to nitrite by soyabean extracts was enhanced by the addition of FAD. FAD was shown to be involved in the transfer of electrons from NADPH to Mo by *N. crassa* nitrate reductase (Nicholas

and Nason, 1954 a, b, c). The enzymatic reduction of exogenous FAD by NADPH was shown to be severely inhibited by the addition of pCMB and reactivated by glutathione indicating the involvement of sulphydryl group(s). This observation has been confirmed with higher plants by many authors (Sanderson and Cocking, 1964; Schrader *et al.*, 1968) and it has recently been proposed (Amy, Garrett and Anderson, 1976) that sulphydryl group(s) are important in the reaction sequence of *N. crassa* nitrate reductase.

Reduced flavins have been shown to be capable of donating electrons to higher plant nitrate reductases (Paneque *et al.*, 1965; Losada *et al.*, 1965) but this activity is not affected by pCMB treatment (Schrader *et al.*, 1968) which inhibits only NADH-dependent activities. It would therefore appear likely that the sulphydryl group(s) are located between the NADH-binding site and the binding site for exogenous flavin.

FAD was identified as a constituent of *Aspergillus nidulans* nitrate reductase by Downey (1971) who was able to observe spectrally the reduction of FAD by NADPH. Reoxidation of the FAD by the addition of nitrate was inhibited by KCN but not by pCMB providing further evidence for the location of the sulphydryl group(s) prior to the FAD-binding site. The presence of FAD in *A. nidulans* nitrate reductase was later confirmed by MacDonald and Coddington (1974).

The involvement of FAD in higher plant and algal (esp. *Chlorella*) nitrate reductases was further suggested from the observations that the presence of FAD protected the NADH-dependent activities (but not the reduced flavin-dependent activities) from thermal denaturation (Zumft *et al.*, 1970; Palacian *et al.*, 1974). Similarly, FAD was found to stabilise barley nitrate reductase during sucrose density gradient centrifugation (Wray and Filner, 1970). FAD has been confirmed as the flavin component of *Chlorella* nitrate reductase by Solomonson *et al.* (1975) and more recently as the flavin component of spinach nitrate reductase (Notton, Fido and Hewitt, 1977; Notton and Hewitt, 1979) using both spectral observations and a specific biochemical test based on the reactivation of apo-D-amino acid oxidase (DeLuca, Weber and Kaplar, 1956).

(c) Cytochrome

The involvement of a cytochrome in assimilatory nitrate reduction was first suggested by Garrett and Nason (1967) who identified a b-type cytochrome, which they designated cyt b_{557}, as a component of *N. crassa* nitrate reductase. Specific reduction of the cyt b_{557} by NADPH and reoxidation by nitrate were demonstrated indicating that the cyt b_{557} was an integral part of the e^- transfer sequence of the enzyme. Downey (1971), however, failed to detect a cytochrome component in the nitrate reductase from *A. nidulans*. In contrast, further work with this same enzyme (MacDonald and Coddington, 1974)

indicated the presence of either a b- or a c-type cytochrome. Recent work from Downey's group (Downey and Steiner, 1979) has indicated the presence of a cyt b_{557} after gel electrophoresis of purified nitrate reductase from *A. nidulans* but as (i) this was not associated with bands possessing reduced methyl viologen-nitrate reductase activity and (ii) the purity of the applied sample was not reported, the significance of these results is, as yet, unclear.

The cytochrome component of *Chlorella* nitrate reductase was shown (Solomonson and Vennesland, 1972, Solomonson *et al.*, 1975) to exhibit the same absorption maxima (at 20°C) as those previously reported for the cyt b_{557} of *N. crassa* nitrate reductase (Garrett and Nason, 1967). These same absorption maxima have also been reported for the cytochrome component of another molybdoenzyme-sulphite oxidase (Cohen and Fridovich, 1971; Kessler and Rajagopalan, 1972). Similar absorption maxima have recently been obtained for the cytochrome component of spinach nitrate reductase (Notton, Fido and Hewitt, 1977) and further work from the same group (Hewitt, Notton and Garner, 1979) has reevaluated these results and concluded that the cytochrome component of spinach nitrate reductase has the same absorption maxima as those reported for the other nitrate reductases with the exception that a slight splitting of the α-band was observed.

In view of all these results it would appear that the nitrate reductases from fungal, algal and higher plant sources are all structurally related in that they all possess flavin, as FAD, and Mo. Also, with the possible exception of *A. nidulans* nitrate reductase they all appear to possess a nearly identical cytochrome component. This similarity is probably an example of conservation during evolution maintaining a structure which has been found to be highly efficient.

4. Arrangement of the Prosthetic Groups in Nitrate Reductase

The likely electron transfer sequence between the components of nitrate reductase is outlined below in Scheme 2.

$$\text{NAD(P)H} \xrightarrow{-SH} \text{FAD} \longrightarrow \text{cyt } b_{557} \longrightarrow \text{Mo} \longrightarrow \text{Nitrate}$$

Scheme 2

Much of the evidence regarding the location of the sulphydryl group(s) has been presented in the previous section. Evidence for the order of the other components comes mainly from work with the nitrate reductase from *N. crassa* (Garrett and Nason, 1967, 1969). The FAD component of this enzyme is freely dissociable with the result that the NADPH-dependent reduction of nitrate can only be observed *in vitro* in the presence of exogenous FAD.

Garrett and Nason (1967) were able to demonstrate that the NADPH-dependent reduction of the cyt b_{557} required the addition of FAD whereas the nitrate-dependent reoxidation of the cytochrome did not require FAD. Thus it may be concluded that the FAD component of the enzyme must be located between the NADPH binding site and the cyt b_{557}.

The direct involvement of Mo at the active site of the enzyme stems from several observations. Firstly, Nicholas and Nason (1954 a,) demonstrated that dithionite-reduced sodium molybdate (to yield Mo^{V}) could donate electrons directly to *N. crassa* nitrate reductase in the absence of added flavin, suggesting that the Mo-site is located nearer to the active site of the enzyme than is the flavin-site. These authors also found evidence for the non-enzymatic reduction of nitrate by the Mo-component and this observation was subsequently confirmed (Nicholas and Stevens, 1955; Ketchum, Taylor and Young, 1976). That Mo compounds can reduce nitrate non-enzymatically strongly suggests that Mo may be involved directly in the enzymic reduction of nitrate by nitrate reductase. Mo is ideally suited for this role (Ochai, 1978) and further support comes from the observation (Garrett and Nason, 1969) that metal-binding agents, e.g. cyanide and azide, inhibit nitrate reduction regardless of electron donor (see below).

5. Molecular Weights of the Nitrate Reductases

When the work to be reported in this thesis was

started there was very little information available in the literature concerning the sizes and structures of higher plant nitrate reductases. Estimates of molecular weight varied greatly from about 160 000 (Hageman and Hucklesby, 1971) to about 500 000 (Anacker and Stoy, 1958) with an apparent average value of about 300 000 (Jolly, Campbell and Tolbert, 1976). Notton, Hewitt and Fielding (1972) determined a value of 240 000 for spinach nitrate reductase by gel filtration through Biogel A0.5 m whilst the same authors (Notton, Icke and Hewitt, 1976) reported a value of 152 000 for the same enzyme determined by sucrose density gradient centrifugation. This diversity in values for the same enzyme illustrates the difficulty in interpreting the data reported in the literature and stems mainly from the fact that all these authors have used only one technique for the determination of molecular weight. For this to be accurate nitrate reductase would have to be a perfectly globular protein which behaved ideally under all test conditions. The conflicting values reported for the molecular weight of spinach nitrate reductase indicate strongly that this is not an ideal protein. Hence any conclusions regarding the structure of higher plant nitrate reductase based on these results must be regarded with caution.

A more reliable and accurate method for the determination of the molecular weight of a protein, even in impure systems, has been reported by Siegel and Monty (1966).

Rather than relying on only one measurement, this method involves the determination of both the sedimentation coefficient of the protein by sucrose density gradient centrifugation and the Stokes radius of the protein by gel filtration. The molecular weight of the protein can be calculated from this data, by means of the equation given in Methods Section IV, together with an indication of the shape of the protein. It is this method which was used throughout the work to be reported in this thesis.

Although no higher plant nitrate reductase had been characterised this way prior to the start of this work, the Siegel and Monty (1966) method had been used to determine accurate molecular weights for the nitrate reductases from *Chlorella* (= 356 000 - Solomonson *et al.*, 1975), *N. crassa* (= 228 000 - Garrett and Nason, 1969) and *A. nidulans* (= 190 000 - MacDonald and Coddington, 1974). It was therefore clear that there were significant differences in the sizes of the nitrate reductases from these sources despite the great similarity between the components of the enzyme (see part (3) of this Introduction) but the reasons for these and their significance in terms of the structures of the enzymes was not clear.

During the past three years our understanding of the nitrate reductases has greatly improved and current ideas and models for the structure of these enzymes will be presented in the General Discussion where they will be compared with the data obtained on barley nitrate reductase

throughout the course of this work.

6. Biochemical Genetics of the Nitrate Reductases

(a) *A. nidulans*

Early genetic work with *A. nidulans* (Cove and Pateman, 1963; Pateman *et al.*, 1964) revealed the presence of six unlinked genes in which mutation resulted in loss of the ability to grow on nitrate as sole nitrogen source. Mutation in any of five of these genes (designated *cnx*) was also shown to result in loss of the ability to grow on hypoxanthine and it was proposed (Cove and Pateman, 1963) that the *cnx* genes coded for a cofactor which was common to both nitrate reductase and xanthine dehydrogenase. One of the *cnx* mutations (*cnx* E) was subsequently shown (Arst, MacDonald and Cove, 1970) to be repairable by growth in the presence of high concentrations of molybdate indicating that the *cnx* E gene product is likely to be responsible for the insertion of Mo into nitrate reductase.

The sixth gene found by Pateman *et al.* (1964) to be essential for growth on nitrate was designated *nia* D and was believed to be the structural gene for nitrate reductase. This was confirmed by MacDonald and Cove (1974) who showed that a temperature-sensitive mutation in *nia* D resulted in the production of a temperature sensitive nitrate reductase. Similarly, *cnx* H was shown to code for

a structural component of nitrate reductase whereas *cnx* E and *cnx* F did not. Temperature-sensitive mutations at the other *cnx* loci (*cnx* ABC and *cnx* G) were not obtained and so could not be analysed.

Based on these genetic studies, MacDonald, Cove and Coddington (1974) proposed that *A. nidulans* nitrate reductase was composed of two *nia* D gene products held together by a Mo-containing cofactor which was coded for by the *cnx* genes.

(b) *N. crassa*

Genetic work with *N. crassa* has not been so detailed as with *A. nidulans* but nevertheless five loci (designated *nit*-1 to *nit*-5) have been identified as essential for growth on nitrate as sole nitrogen source. In a series of papers (Sorger, 1965, 1966; Sorger and Giles, 1965) it was shown that two of these genes, *nit*-1 and *nit*-3 coded for structural components of *N. crassa* nitrate reductase. The *nit*-1 gene was thought to code for a polypeptide which was responsible for the transfer of electrons from Mo to nitrate (analogous to the product of the *cnx* genes of *A. nidulans*) whilst the *nit*-3 gene was thought to code for the rest of the nitrate reductase molecule (analogous to the product of the *nia* D gene of *A. nidulans*).

These observations were confirmed and extended (Nason *et al.*, 1970, 1971; Ketchum *et al.*, 1971) by the

demonstration that nitrate reductase activity could be reconstituted *in vitro* by the mixing of extracts from *nit*-1 and *nit*-3 mutants. Subsequent work (Nason *et al.* 1974) demonstrated that the *nit*-3 extract could be replaced by a Mo-containing component (MCC) derived by acid treatment from any of several Mo-containing enzymes tested. It could therefore be concluded that the *nit-1* gene specifies a Mo-containing component which is common to several molybdoenzymes and is analogous to the *cnx* genes of *A. nidulans*. It is, however, surprising that only one *nit*-1 gene has been identified as there are five *cnx* genes required in *A. nidulans* for the manufacture of MCC which has been shown (Lee *et al.*, 1974) to be dialysable, to have a molecular weight of only about 1000 and to require added Mo for activity. It was therefore proposed by these authors that *N. crassa* nitrate reductase was composed of *nit*-3 gene products bound together by the small *nit*-1 gene product (MCC). This model is identical to that proposed for *A. nidulans* nitrate reductase by MacDonald and Coddington (1974).

(c) Algae

The most studied algal nitrate reductase is that from *Chlorella* but no genetic analysis has been undertaken with this species. However, two groups have reported genetic studies with another green alga, *Chlamydomonas reinhardii*. Both groups (Nichols and Syrett, 1978; Nichols, Shehata

and Syrett, 1978; Sosa, Ortega and Barea, 1978) reported the existence of mutations analogous to the *cnx* (*nit*-1) and *nia* D (*nit*-3) mutations of *A. nidulans* (*N. crassa*) but insufficient data is available to allow definite conclusions to be drawn.

(d) Higher Plants

Genetic studies are made difficult in higher plants by the fact that these are not haploid and so any recessive mutation is masked. Despite these problems, nitrate-reductase-negative mutations have been obtained in *Nicotiana tabacum* (Müller *et al.*, 1976) and some of these have been shown to also lack xanthine dehydrogenase activity (Mendel and Müller, 1976) providing evidence that these enzymes share a common cofactor in higher plants as well as in fungi.

Mutants lacking nitrate reductase activity have also been obtained in barley (Warner, Lin and Kleinhofs, 1977) but insufficient data was reported to allow these to be evaluated.

7. Determination of the Physiological Reductant of Higher Plant Nitrate Reductase

Nitrate reductase activity was first demonstrated in a higher plant by Evans and Nason (1953) using extracts from soybean. These authors found that both NADH and

NADPH were capable of donating electrons to the enzyme and a stoichiometric relationship between number of moles of reduced pyridine nucleotide oxidised and number of moles of nitrite produced was determined. In contrast, Stoy (1956) found that reduced riboflavin was a more efficient electron donor to wheat nitrate reductase than was NADH. However, Beevers, Flesher and Hageman (1964) showed that the nitrate reductase in 15 out of 16 plant species tested was specific for, or had a preferential requirement for, NADH whilst the nitrate reductase from soybean was found to be able to utilise NADH or NADPH, confirming the results of Evans and Nason (1953).

In spite of these results, Paneque *et al.* (1965), using 130-fold purified spinach nitrate reductase, proposed that reduced FMN and FAD were the natural reductants for nitrate reductase and that the nitrate reductase activity previously described by other workers was, in fact, two enzymes - an NADP reductase and a reduced-flavin nitrate reductase. In a companion paper Losada *et al.* (1965) reported nitrate reduction by a reconstituted spinach chloroplast system in which FAD was reduced by illuminated grana. However, it was later shown by the same authors (Paneque and Losada, 1966) that their spinach nitrate reductase could utilise NADH as electron donor without the requirement of added cofactors, whereas in their previous reports the addition of both FMN and an NADP reductase was required for activity.

Schrader *et al.* (1968) subsequently reported that NADH and FMNH were both effective electron donors for the nitrate reductases from maize, marrow and spinach, provided that these cofactors were supplied at their optimum concentrations. However, the required concentration of FMNH was 40-100 fold higher than the required concentration of NADH indicating that the *in vivo* reductant was likely to be NADH. Also NADPH was only functional when supplied together with an NADP reductase and exogenous FMN to give enzymatic generation of FMNH. All attempts to separate NADH- and FMNH- nitrate reductase activities were unsuccessful and it was concluded that nitrate reductase was a single moiety with the ability to use either NADH or FMNH as cofactor.

The ability of the nitrate reductase from soybean and from some genotypes of corn to utilise NADH or NADPH as electron donor was examined by Wells and Hageman (1974) who reported that the two activities could not be separated by either isoelectric focusing or DEAE-cellulose chromatography, suggesting that the two activities were possessed by the same protein. The ability to use NADPH as source of electrons was correlated by these authors with the level of NADPH-phosphatase activity in the extract. Addition of KH_2PO_4 and KF, which inhibit NADPH-phosphatase activity in *in vitro* assays, was shown to abolish NADPH-nitrate reductase activity, suggesting that NADH is the natural electron donor even in these tissues. These results therefore indicate that NADH should be the *in vivo* reductant of

all higher plant nitrate reductases.

However, conflicting results have been more recently obtained by Jolly, Campbell and Tolbert (1975, 1976) who achieved separation of the NADH- and NADPH-nitrate reductases from soybean by DEAE-cellulose chromatography and gel filtration. NADH-nitrate reductase had an estimated (solely by gel filtration) molecular weight of 330 000 whilst the NADPH-nitrate reductase was much smaller with a molecular weight of only 220 000. Similar results have been obtained with extracts from rice by Shen, Funkhouser and Guerrero (1976) who reported partial separation of NADH- and NADPH-nitrate reductase activities by Blue Dextran-Sepharose chromatography.

Very recent work on the two nitrate reductase activities from soybean (Iranzo and Campbell, 1979) has indicated that they could be separated by Blue-Sepharose chromatography. The two activities were also shown to reach maxima at different plant ages: NADH-dependent activity was highest 8 days after the seeds were imbibed whereas the NADPH-dependent activity peaked 10 days after the seeds were imbibed by which time the NADH-dependent activity had decreased by 15-20%. A possible explanation for these results will be given in the General Discussion.

The nitrate reductases have been classified by the Enzyme Commission in relation to their *in vivo* reductants. Thus, NADH is the must common electron donor to higher

plant nitrate reductases and enzymes catalysing the NADH-dependent reduction of nitrate to nitrite are classified as EC 1.6.6.1. In contrast to the higher plant enzyme, fungal nitrate reductases can utilise only NADPH and are classified as EC 1.6.6.3. There are some nitrate reductases, notably from algal sources, which can utilise NADH or NADPH. These are denoted the NAD(P)H nitrate reductases and are classified as EC 1.6.6.2.

8. Reactions Catalysed by the Nitrate Reductases

(a) Electron Donors

As indicated above, the physiological reductants of the assimilatory nitrate reductases are either NADH or NADPH. However, other electron donors and acceptors have been found to interact with these enzymes making it possible to assay different parts of the nitrate reductase molecule.

Stoy (1956) was the first to show that reduced flavins could donate electrons to nitrate reductase and, as indicated in the previous section, some authors (Paneque *et al.*, 1965; Losada *et al.*, 1965) believed them to be the physiological electron donors. Schrader *et al.* (1968) demonstrated that whereas pCMB treatment resulted in the loss of NADH-nitrate reductase activity, the FMNH-nitrate reductase activity was unaffected indicating that FMNH donates electrons to a site beyond the sulphydryl group(s) of the enzyme. Similarly, Garrett and Nason (1969) have

shown that, unlike NADPH-nitrate reductase activity, FMNH-nitrate reductase activity is not enhanced by addition of FAD indicating that FMNH probably donates electrons to a site beyond FAD. Heat inactivation studies on nitrate reductases (Garrett and Nason, 1969; Wray and Filner, 1970) have shown that, whereas NAD(P)H-dependent activities are heat-labile, the FMNH-dependent activities are heat-stable, providing further support that these reductants donate electrons to different portions of the nitrate reductases.

In addition to reduced flavins, it has also been shown that reduced viologen dyes are capable of donating electrons to the nitrate reductases from squash, maize and spinach (Hageman, Cresswell and Hewitt, 1962; Cresswell *et al.*, 1965), *N. crassa* (Garrett and Nason, 1969), *A. nidulans* (MacDonald and Coddington, 1974) and *Chlorella* (Zumft *et al.*, 1969). In view of these results, reduced viologen dyes are now considered almost universally effective as electron donors for the nitrate reductases. Like FMNH-nitrate reductase, reduced viologen-nitrate reductase is not susceptible to pCMB treatment or thermal inactivation under conditions where NADH-dependent activities are rapidly lost. Thus FMNH and reduced viologens both appear to donate electrons to the terminal portion of nitrate reductase. However, evidence has been presented (Garrett and Nason, 1969) which indicates that reduced viologen nitrate reductase activity is more stable

than FMNH-nitrate reductase activity. This suggests that reduced viologens donate electrons to a site closer to the nitrate-reducing site than that to which FMNH donates electrons. This fact, together with the high electronegative potential of reduced viologens, has prompted many authors (Hewitt, Hucklesby and Notton, 1976) to believe that reduced viologens donate electrons directly to the Mo site of the nitrate reductases.

(b) Electron Acceptors

In addition to the physiological substrate, nitrate, several other electron acceptors have been found to react with the nitrate reductases. Kinsky and McElroy (1955) first noted the ability of *N. crassa* nitrate reductase to catalyse the NADPH-dependent reduction of exogenous cytochrome c. This was subseqeuntly confirmed in other species (Cove and Coddington, 1965; Paneque and Losada, 1966) and it was proposed (Sorger, 1964, 1965, 1966; Sorger and Giles, 1965) that *N. crassa* nitrate reductase was composed of two closely associated polypeptides, one transferring electrons from NADPH to FAD (and catalysing NADPH-cytochrome c reductase activity) and the other accepting electrons from the reduced FAD in the first polypeptide, passing them to Mo, and from there to nitrate. (Note: the existence of the cytochrome component was not recognised at this time.)

In all cases, (Garrett and Nason, 1969; Zumft *et al.*,

1969; Wray and Filner, 1970) NAD(P)H-cytochrome c reductase activity was found to be sensitive to sulphydryl reagents and elevated temperatures, as was the NAD(P)H nitrate reductase activity but not the FMNH-nitrate reductase activity. These results suggest that NADH-cytochrome c reductase activity is a measure of the initial portion of the electron transfer sequence of nitrate reductase and is often referred to as either the 'dehydrogenase' activity or the 'diaphorase' activity of nitrate reductase.

Cytochrome c, however, is not the only electron acceptor capable of interacting with nitrate reductase Others include ferricyanide, tetrazolium salts (Pateman, Rever and Cove, 1967) and DCPIP (Nicholas and Nason, 1954b). The NADPH-dependent reduction of DCPIP by *N. crassa* nitrate reductase was shown to require FAD and to be susceptible to pCMB treatment and is therefore likely to be the same activity as measured with cytochrome c as substrate. Higher plant nitrate reductases are also thought (Hewitt, 1975; Notton, Fido and Hewitt, 1977) capable of donating electrons to ferricyanide, DCPIP and tetrazolium salts but the evidence for this is rather circumstantial.

9. Sites of Interaction of Electron Donors and Acceptors with the Nitrate Reductases

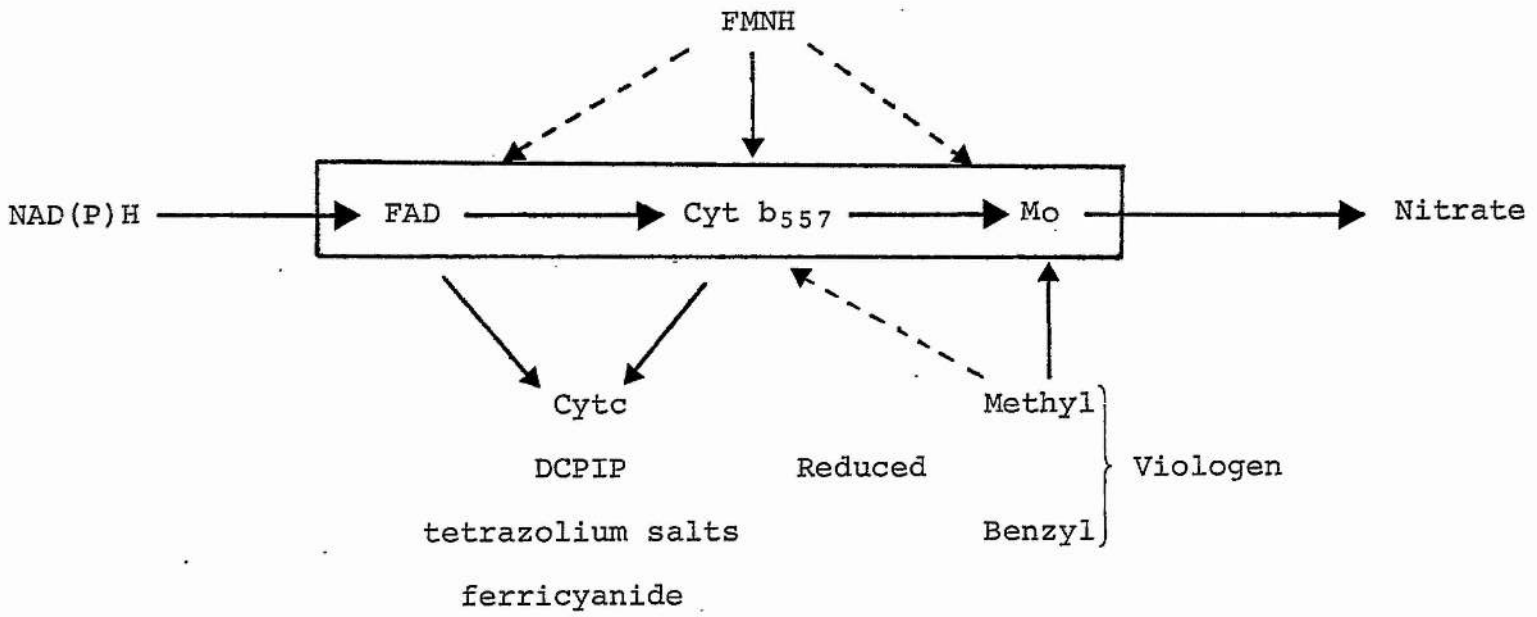
The probable sites of interaction of electron donors and acceptors with the nitrate reductases is outlined in

Scheme 3 from which it is obvious that a great deal of uncertainty exists. The major controversy (Hewitt, 1974; Hewitt, 1975; Notton, Fido and Hewitt, 1977; Fido *et al.*, 1979) regards the site at which cytochrome c and the other acceptors interact with nitrate reductase. Reduction of all these substrates has been shown to involve the sulphydryl group(s) and FAD and so the FAD is the earliest point at which electron donation to these substrates can take place whilst it is also possible that electrons are donated to these substrates via the cyt b_{557} component of the enzyme. Comparison with the sequence of components of the mitochondrial respiratory chain indicates that the latter possibility is perhaps the most likely.

The results of heat inactivation studies and treatment with sulphydryl reagents are compatible with either site of interaction if it is assumed that electron donation by NAD(P)H is most sensitive to these treatments. Thus, loss of NAD(P)H cytochrome c activity does not necessarily imply that the site at which cytochrome c accepts electrons has been affected. Evidence that the cyt b_{557} component of nitrate reductase is the site at which the electron acceptors interact has been presented by Notton, Fido and Hewitt (1977) who showed that NADH-reduced cyt b_{557} could be reoxidised by the addition of DCPIP. Thus DCPIP can either interact directly with the cyt b_{557} or the reoxidation was due to reverse electron flow through the enzyme to the site at which DCPIP did

SCHEME 3

POSSIBLE SITES OF INTERACTION OF SUBSTRATES AND ELECTRON DONORS WITH THE NITRATE REDUCTASES



interact.

It is therefore not possible to assign a precise site for the interaction of the 'dehydrogenase electron acceptors' as the data available can be interpreted in different ways. Further work still needs to be done in order to find a solution to this problem.

Evidence relating to the sites of interaction of the electron donors has been presented in the previous section of this Introduction.

10. Induction of Nitrate Reductase Activity

The formation of nitrate reductase activity in response to nitrate was first noted with *N. crassa* by Kinsky and McElroy (1955) who noted that NADPH-nitrate- and NADPH-cytochrome c reductases were induced in parallel. The ability of higher plants to induce nitrate reductase activity was first reported by Tang and Wu (1957) who demonstrated that plants grown in buffer lacking nitrate also lacked nitrate reductase activity whereas plants exposed to nitrate for 5 days showed appreciable levels of nitrate reductase activity. A much faster response to nitrate was reported by Afridi and Hewitt (1959) using small leaf segments of cauliflower, white mustard and sunflower. Nitrate reductase activity was shown to appear after a lag of 1 hour following vacuum infiltration of nitrate into leaves which had been grown in the presence

of ammonium and therefore lacked nitrate reductase activity. A similar lag was observed when Mo was vacuum infiltrated into Mo-deficient, nitrate grown leaves. As noted by Filner, Wray and Varner (1969) these results indicated that nitrate and Mo were involved in the control of nitrate reductase synthesis. Had Mo merely activated a pre-existing apoenzyme, a difference in kinetics would most likely have been found.

However, such a difference in kinetics has recently been demonstrated (Jones *et al.*, 1978) in Paul's Scarlet Rose suspension cultures. These authors observed a lag of about 30 minutes before nitrate reductase activity appeared after addition of nitrate to Mo-sufficient cells but observed no lag upon addition of Mo to Mo-deficient cells. Induction by nitrate appeared to involve mRNA-dependent synthesis of apoprotein followed by rapid activation with Mo in intact cells, independently of protein synthesis.

The molecular basis of nitrate reductase induction has also been studied in other organisms. Using different combinations of cycloheximide and actinomycin D, it has been demonstrated (Subramanian and Sorger, 1972; Sorger and Davies, 1973) that nitrate is essential for translation of NADPH-nitrate reductase mRNA species, but not for the transcription of the nitrate reductase gene(s) in *N.crassa*. Conflicting results have been obtained with the yeast *Candida utilis* by Choudary and Rao (1976a, b) who

found that nitrate induced the capacity to synthesise nitrate reductase during transcription and was not obligatory during translation. Both sets of authors found that nitrate was essential to maintain the stability of nitrate reductase following its formation.

Nitrate reductase has been shown (Ingle, Joy and Hageman, 1966) to be inducible by nitrite even in the presence of ammonium and Shen (1972) has demonstrated induction of nitrate reductase with chloramphenicol in rice. However, induction with choramphenical was shown to result in predominance of NADPH-nitrate reductase activity whereas induction with nitrate resulted in a predominance of NADH-nitrate reductase activity. Both the NADH- and NADPH-nitrate reductases co-sedimented during sucrose density gradient centrifugation (Shen 1972).

11. Repression of Nitrate Reductase Activity

Repression of nitrate reductase activity by ammonium was first shown by Kinsky (1961) in *N. crassa* and by Morris and Syrett (1963) in *Chlorella*. These results were confirmed with the nitrate reductase from *A. nidulans* by Cove (1966) who also showed that removal of nitrate from the medium could lead to repression of nitrate reductase. No effect of ammonium on nitrate reductase activity could be detected *in vitro* as was later confirmed for *N. crassa* nitrate reductase by Garrett and Nason (1969).

Attempts (Afridi and Hewitt, 1964; Beevers *et al.*, 1965) to demonstrate ammonium repression of higher plant nitrate reductase were unsuccessful. Filner (1966), however, reported that certain amino acids and casein hydrolysate could lead to repression (or inactivation) of the nitrate reductase in tobacco XD cell suspension cultures while other amino acids were found to derepress nitrate reductase.

Nitrogen metabolite repression of *A. nidulans* nitrate reductase has been extensively reviewed by Arst and MacDonald (1973) who showed that glutamate auxotrophs were essentially insensitive to ammonium repression, suggesting that glutamate dehydrogenase (the major ammonium-assimilatory enzyme in fungi - see Dantzig, Wiegmann and Nason, 1979) or glutamate is involved in the *in vivo* regulation of *A. nidulans* nitrate reductase. A recent report (Prekumar, Sorger and Gooden, 1979) gives similar results and suggests that glutamine is the main co-repressor of nitrate reductase in *N. crassa*.

Very recently, Dunn-Coleman and Garrett (1979), using a glutamine synthetase mutant (*gln* - 1a) of *N. crassa*, have obtained evidence which suggests that nitrate reductase is regulated by the oligomeric state of glutamine synthetase, which is a function of nitrogen-sufficiency. An octameric form of glutamine synthetase predominates in the presence of excess ammonium while a tetrameric form predominates under conditions of nitrogen limitation

(Davilla *et al.*, 1978). The results of Dunn-Coleman and Garrett (1979) suggest that the 'ammonium repression' of nitrate reductase in *N. crassa* is mediated by the glutamine synthetase octamer.

The results obtained with higher plants (Beevers *et al.*, 1965) suggest that ammonium-repression is not a major controlling factor in higher plants. It has been recently proposed (Solomonson and Spehar, 1977) that higher plant nitrate reductases are instead regulated by a system of reversible inactivation in which the enzyme is inactivated by binding to both NADH and cyanide and reactivated by some, as yet unidentified, oxidant. This will be examined in much greater detail later in this thesis.

12. Subcellular Location of Higher Plant Nitrate Reductase

Evans and Nason (1953) demonstrated that illuminated chloroplast grana could provide electrons for nitrate reduction, but the chloroplast grana were themselves unable to reduce nitrate unless a purified nitrate reductase was added. These authors therefore concluded that nitrate reductase was either (i) not located in the chloroplast, or (ii) was lost from the chloroplast during extraction. These observations were later confirmed by both Ramirez *et al.* (1964) and Swader and Stocking (1971). However, Grant, Canvin and Atkins (1970) showed that chloroplasts with intact outer membranes and which showed high rates of CO_2-fixation contained at least some nitrate- and

nitrite reductase. However, calculations on the distribution of these enzymes compared with the distributions of pyruvate kinase and cytochrome c oxidase, prompted the authors to conclude that most of the nitrate- and nitrite-reductase activities were located outside the chloroplast.

In attempts to overcome leakage of enzymes from the chloroplasts during extraction Ritenour *et al.* (1967) and Coupé, Champigny and Moyse (1967) used non-aqueous techniques for the isolation of chloroplasts from corn, foxtail grass and barley. Basing their results on the distribution of clorophyll, Coupé, Champigny and Moyse (1967) concluded that nitrate reductase was located within the chloroplast, while Ritenour *et al.* (1967), by comparison with the distribution of marker enzymes, concluded that nitrate reductase was a cytoplasmic enzyme. These authors did not discount the possibility that nitrate reductase may have been located on the outer chloroplast membrane which is lost during non-aqueous extraction procedures. The location of nitrate reductase on the outer chloroplast membrane has been suggested (Eaglesham and Hewitt, 1971) based on the observed inhibition of nitrate reductase by ADP. Hewitt (1975) has argued that this membrane location for nitrate reductase would explain the observed involvement of phytochrome in the induction of nitrate reductase (Jones and Sheard, 1972; 1973) in etiolated pea leaves.

Wallsgrove, Lea and Miflin (1979) obtained no evidence

to support this membrane location for nitrate reductase. Using ruptured isolated pea protoplasts, in which 80-90% of the chloroplasts remained intact, only 5% of the total nitrate reductase was found associated with the chloroplasts. These results indicate that either nitrate reductase is only very loosely associated with the outer chloroplast membrane or nitrate reductase is a true cytoplasmic enzyme, in which case the 5% of the total nitrate reductase activity found associated with the chloroplasts would be due to non-specific adsorption during isolation of the chloroplasts. This is supported by the results of Dalling, Tolbert and Hageman (1972a, b) who concluded that nitrate reductase is a cytoplasmic enzyme.

It would therefore appear likely that if higher plant nitrate reductase is associated with the chloroplast then this association must be very loose and can be destroyed by even the mildest of extraction procedures. This lack of evidence for a membrane location for nitrate reductase is perhaps rather surprising as the next step in the assimilation of nitrate, the reduction of nitrite to ammonium by nitrite reductase, occurs exclusively within the chloroplast (Ritenour *et al.*, 1967; Wallsgrove, Lea and Miflin, 1979) and so nitrite, the product of nitrate reductase, must be transported into the chloroplast prior to reduction. Indeed, as will be described in the next section, the reducing power necessary for the activity of nitrate reductase is likely to be derived from the chloroplast.

Hence it would seem logical that nitrate reductase should be associated with the chloroplast but the experimental results do not support this.

13. Source of Reducing Power for Higher Plant Nitrate Reductase

The reduction of nitrate is an energy-requiring process and it was initially thought (Evans and Nason, 1953) that this requirement was met directly by photosynthesis, as reduced pyridine nucleotides are formed during non-cyclic photophosphorylation. This was supported by the results of Losada *et al.* (1965) and Paneque *et al.* (1965) who contended that reduced flavin nucleotides were the natural reductants of nitrate reductase and that these were reduced directly by the chloroplast.

However, in view of the fact (Beevers, Flesher and Hageman, 1964) that NADH is the natural reductant of higher plant nitrate reductase whilst it is NADPH which is formed during non-cyclic photophosphorylation (Ogren and Krogmann, 1966) it is likely that the reducing power for nitrate reduction does not come directly from the chloroplast. This conclusion is further supported by the observation (Heber and Santorius, 1965) that the inner chloroplast membrane is relatively impermeable to pyridine nucleotides.

It has been suggested (Stocking and Larson, 1969;

Walker and Crofts, 1970) that this problem may be overcome if NADH is generated in the cytoplasm from intermediates synthesised in the chloroplast as a result of photosynthetic carbon-fixation. Klepper and Hageman (1969), using leaf discs vacuum infiltrated with nitrate and incubated under anaerobic conditions, were able to demonstrate an enhancement of nitrite production when the infiltration medium was supplemented with sugars (fructose-1,6-diphosphate and 3-phosphoglycerate) but not when supplied with organic acids (citrate, α-oxoglutarate or succinate). The failure of the organic acids to stimulate nitrate reduction argues against the possibility that the reductant for nitrate reduction was generated through the tricarboxylic acid cycle. In subsequent studies Klepper, Flesher and Hageman (1971) suggested that some product of photosynthesis (3-phosphoglycerate, hexoses or phosphorylated hexoses) migrated from the chloroplast to the cytoplasm where they were metabolised by the glycolytic enzymes to generate NADH.

These results have recently been confirmed and extended by Mann, Hucklesby and Hewitt (1978) who demonstrated the ability of glycolytic intermediates to enhance nitrite production in spinach leaf discs. The authors were unable to demonstrate any stimulation of nitrite production by the addition of glycollate indicating that photorespiration is not a major source of reducing power for nitrate reduction. This conflicts with previous

suggestions (Lips, 1971; Plaut and Littan, 1974) that photorespiration was a major source of reducing power for nitrate reduction.

Mann, Hucklesby and Hewitt (1978) also demonstrated a stimulatory effect of malate on nitrite production, confirming the previous results of Neyra and Hageman (1976) with corn seedlings. The ability of malate to act as source of reductant for nitrate reduction suggests that the malate / oxaloacetate shuttle between chloroplast and cytoplasm (Walker, 1974; Heber, 1964) may be involved and this possibility has recently been studied by Rathnam (1978) in spinach leaf protoplasts. Nitrate reduction was shown to be stimulated 3- to 4-fold in the presence of HCO_3^- and a similar stimulation was found in the absence of CO_2-fixation by the addition of malate, oxaloacetate, 3-phosphoglycerate or dihydroxyacetone-phosphate (DHAP). Stimulation by malate or DHAP was independent of light but that by oxaloacetate or 3-phosphoglycerate was light dependent. Reduction of nitrate was shown to be linked to the cytoplasmic oxidation of DHAP or malate and it was demonstrated that both the malate / oxaloacetate and 3-phosphoglycerate/DHAP shuttles could support CO_2-fixation and/or nitrate reduction. With this system, the stoichiometric assimilation of nitrate into α-amino nitrogen was demonstrated.

An alternative view has recently been proposed (Sawhney, Naik and Nicholas, 1978a, b) which accounts

for the observation (Canvin and Atkins, 1974) that *in vivo* nitrate reduction is totally dependent upon light. In this model, the NADH required for nitrate reduction is generated by the tricarboxylic acid cycle, in direct contrast to the results of Klepper and Hageman (1969). It is envisaged that light-driven photosynthesis leads to an elevation of the cytoplasmic energy charge which, when transmitted to mitochondria, inhibits the oxidation of NADH by the electron transfer chain, thus making the NADH available for nitrate reduction. Although the data presented by these authors is inconclusive, further support for their proposals has recently been presented by Canvin and Woo (1979).

If view of these conflicting ideas it is not possible to state categorically from which source the reducing power for nitrate reduction in higher plants is derived.

14. Aims of this Work

The aims of the work reported in this thesis were:

(i) to purify barley nitrate reductase and to determine its physical properties, subunit composition and prosthetic group composition.

(ii) to examine the low molecular weight NADH-cytochrome c reductase species (Wray and Filner, 1970) and to discover whether it was related to nitrate reductase

(iii) to examine the interaction of barley nitrate reductase with alternative electron acceptors and donors and thereby determine their possible sites of interaction with the enzyme

(iv) to determine whether barley NADH-nitrate reductase activity could be reconstituted from the low molecular weight NADH-cytochrome c reductase species (Wray and Filner, 1970) by the addition of a Mo-containing component (Rucklidge, Notton and Hewitt (1976), and

(v) to characterise the ferrocyanide-activated NADH-cytochrome c reductase species (Wallace and Johnson, 1978) and to determine the relationship of this species to nitrate reductase.

MATERIALS

CHEMICALS

The following were all obtained from the Sigma London Chemical Company:

Alcohol dehydrogenase (yeast); Bovine serum albumin (Fraction V); Catalase (bovine liver); Cysteine (free base); Cytochrome c (horse heart, type III); Dithiothreitol; FAD (grade III); Ferritin (horse spleen, type I); Fibrinogen (bovine, fraction I, type IV); FMN (sodium salt, synthetic); HEPES; Methylene bis-acrylamide; Methyl viologen; Myoglobin (whale skeletal muscle, type II); NAD^+ (yeast, grade IV); NADH (yeast, Grade III); Nitroblue tetrazolium (grade III); Streptomycin sulphate; 2,3,5-Tetra zolium chloride and Urease (Jack beans, type IX).

Sephadex G25, Sephadex G200, CNBr-activated Sepharose 4B, Blue Sepharose CL-6B and Blue Dextran 2000 were obtained from Pharmacia, Sweden.

Biogel A0.5 m and Biogel A1.5 m were obtained from Biorad Laboratories Ltd., Watford, Hertfordshire.

DEAE-Cellulose was obtained from Whatman Biochemicals Ltd., Maidstone, Kent.

All other chemicals were of the highest grade available from the usual commercial sources.

METHODS

SECTION I - GROWTH OF PLANT MATERIAL

Barley (*Hordeum vulgare* cv. Golden Promise) was used throughout this work. Seeds were sown thickly in trays containing vermiculite, watered with tap water and placed in the dark at 28°C to germinate. After 24 hours, 48 hours, and 72 hours the trays were watered with a modified half-strength Hoagland nutrient solution containing 15.8 mM KNO_3 (Table 1). For plants grown in the absence of nitrate, an analogous nutrient solution lacking nitrate was used.

After 64 hours of growth in the dark, the trays were transferred to continuous light, (1000 lux) supplied by Gro-Lux fluorescent tubes, at 25°C. Plants were routinely harvested after 24 hours in the light, i.e. after 88-90 hours of growth by which time the average length of the shoots was 4 cm.

For experiments requiring older plants (see Results Section) the period of dark growth was unaltered and the plants were maintained under continuous illumination until use. During this period they received additional nutrient solution at 24 hour intervals.

SECTION II - EXTRACTION AND PURIFICATION PROCEDURES

Extraction of Plant Material

Shoots were harvested by cutting just above the seed

TABLE 1

COMPOSITION OF HALF-HOAGLAND NUTRIENT SOLUTION

	g/litre
Stock Solution A	
NaFe EDTA	38.44
Stock Solution B	
KH_2PO_4	34.25
$MgSO_4.7H_2O$	126.65
$ZnSO_4.7H_2O$	0.0555
$MnSO_4.5H_2O$	0.0206
H_3BO_3	0.725
$Na_2MoO_4.2H_2O$	0.00622

The working solution consisted of 10 ml of Stock Solutions A and B in 5 litres of distilled water. 8 g of KNO_3 were then added to give a final nitrate concentration of 15.8 mM. KNO_3 was omitted if nitrate-less Half-Hoagland nutrient solution was required.

and then ground in a chilled mortar and pestle with cold 0.05 M potassium phosphate buffer pH 7.5 containing 0.1 mM EDTA, 10 μM FAD and 1 mM cysteine (Buffer I). The cysteine was always added to the buffer immediately prior to use and the ratio of buffer to plant material was always 3 ml of buffer per gram fresh weight of shoots.

When small amounts of tissue were extracted the brei was immediately centrifuged at 38 000 g in an MSE High Speed 18 Refrigerated centrifuge for 20 min at 3°C, and the resulting supernatant used as source of enzyme. For larger amounts of tissue, which were used during purification procedures, the brei was filtered through a double layer of muslin and the resultant filtrate was taken as source of enzyme for the determination of initial specific activity. The filtrate was then subjected to streptomycin sulphate treatment (see below).

Streptomycin Sulphate Treatment

Nucleic acid material was removed by the addition of streptomycin sulphate to the filtered extract prior to centrifugation at a rate of 5 mg per gram fresh weight of tissue extracted (Notton, Fido and Hewitt, 1977). After stirring at 4°C for 5 min the extract was then centrifuged at 40 000 g for 45-60 min at 3°C in either an MSE High Speed 18 centrifuge or an MSE High Speed 25 Centrifuge. Precipitated material was discarded and the supernatant used as a source of enzyme.

Ammonium Sulphate Fractionation

Ammonium sulphate fractionation of extracts was routinely carried out by the slow addition of the required amount of solid ammonium sulphate (Dawson, Elliot and Jones, 1969) to the extract which was stirred throughout over an ice-water bath. After the addition was completed the solution was restored to pH 7.5 by the addition of dilute ammonium hydroxide.

When, however, protein was being precipitated from a solution of enzyme in 40% (v/v) glycerol (the form in which enzyme was stored - see later), ammonium sulphate fractionation was carried out by the addition of the required amount of a saturated solution of ammonium sulphate pH 7.5, as determined by the equation

$$V = \frac{100(S_2-S_1)}{1 - S_2}$$

where S_1 is the initial ammonium sulphate concentration and S_2 is the final ammonium sulphate concentration. S_1 and S_2 are expressed as fractions of saturation. (Dawson Elliot and Jones, 1969).

After either treatment the solution was stirred at 4°C for 20 min following which precipitated protein was collected by centrifugation at 20 000 g for 30 min at 3°C using an MSE High Speed 18 centrifuge. The resulting precipitate was then dissolved in the minimum amount of Buffer I.

Biogel A1.5 m Chromatography

Biogel A1.5 m 100-200 mesh was packed into a column (4.1 cm × 108 cm) and equilibrated in Buffer I lacking cysteine (Buffer II). Omission of cystein from the buffer allows NADH-cytochrome c reductase activity to be assayed in the fractions (see later). 15-20 ml of enzyme sample, derived from ammonium sulphate fractionation, were applied to the column which was then eluted with Buffer II. As the void volume of this column was approximately 500 ml, 450 ml of buffer was allowed to pass through the column before fractions (15 ml) were collected either by means of a Copley Fractomin fraction collector or an LKB Ultrorac fraction collector. All column operations were carried out at 4 °C.

DEAE-Cellulose Chromatography

Samples for application to DEAE-cellulose were first desalted by passage through a column of Sephadex G25. Column and sample sizes varied with experiments, but enzyme was routinely applied to the column either in Buffer I or in Buffer II when NADH-cytochrome c reductase activity was to be determined (see later).

The column was then washed with the same buffer until the absorbance of the eluate at 280 nm had dropped to below 0.1. A linear gradient of from 50 mM to 300 mM potassium phosphate buffer pH 7.5, containing 10 μM FAD and 0.1 mM EDTA (and 1 mM cysteine if Buffer I had been used)

was then applied to the column in order to elute the required enzymic activity. All column operations were carried out at 4°C.

Affinity Chromatography with Blue-Sepharose and Blue-Dextran Sepharose

Column sizes and eluant details will be given in the Results section. With commercially available Blue-Sepharose (Pharmacia) all column operations were carried out with the required eluants contained in Buffer I. With the Blue-Dextran Sepharose, however, all column operations were carried out with the required eluants contained in 20 mM potassium phosphate buffer pH 7.5, containing 0.1 mM EDTA, 10 µM FAD and 1 mM cysteine (Buffer III). All operations were carried out at 4°C.

SECTION III - ENZYME ASSAYS

NADH - Nitrate Reductase

This was assayed by the method of Wray and Filner (1970). Present in the reaction were 0.5 ml of 0.1 M potassium phosphate buffer pH 7.5, 0.1 ml of 0.1 M potassium nitrate, 0.1 ml of 0.1 mM NADH, enzyme sample and distilled water to give a final volume of 1 ml. The reaction was started by the addition of enzyme sample and after incubation at 25°C for a suitable time period (10 min for crude extracts) the reaction was terminated by

the addition of 1 ml of 1% (w/v) sulphanilamide in 3 M HCl followed by 1 ml of 0.02% (w/v) N-1-naphthylethylene-diaminedihydrochloride (Snell and Snell, 1949). Precipitated protein was removed by centrifugation at top speed on a bench-top centrifuge for 5 min.

The absorbance of this solution at 540 nm was then read against a control in which the sulphanilamide solution had been added prior to enzyme sample. Absorbance at 540 nm was converted to nitrite production by means of a previously established calibration plot of 0 - 100 nmoles nitrite (Fig. 1). Where NADH-nitrate reductase activity was estimated in samples not containing cysteine (e.g. in column fractions for which NADH-cytochrome c reductase activity was also to be measured) 1 mM cysteine was included in the assay mixture as suggested by Wray and Filner (1970).

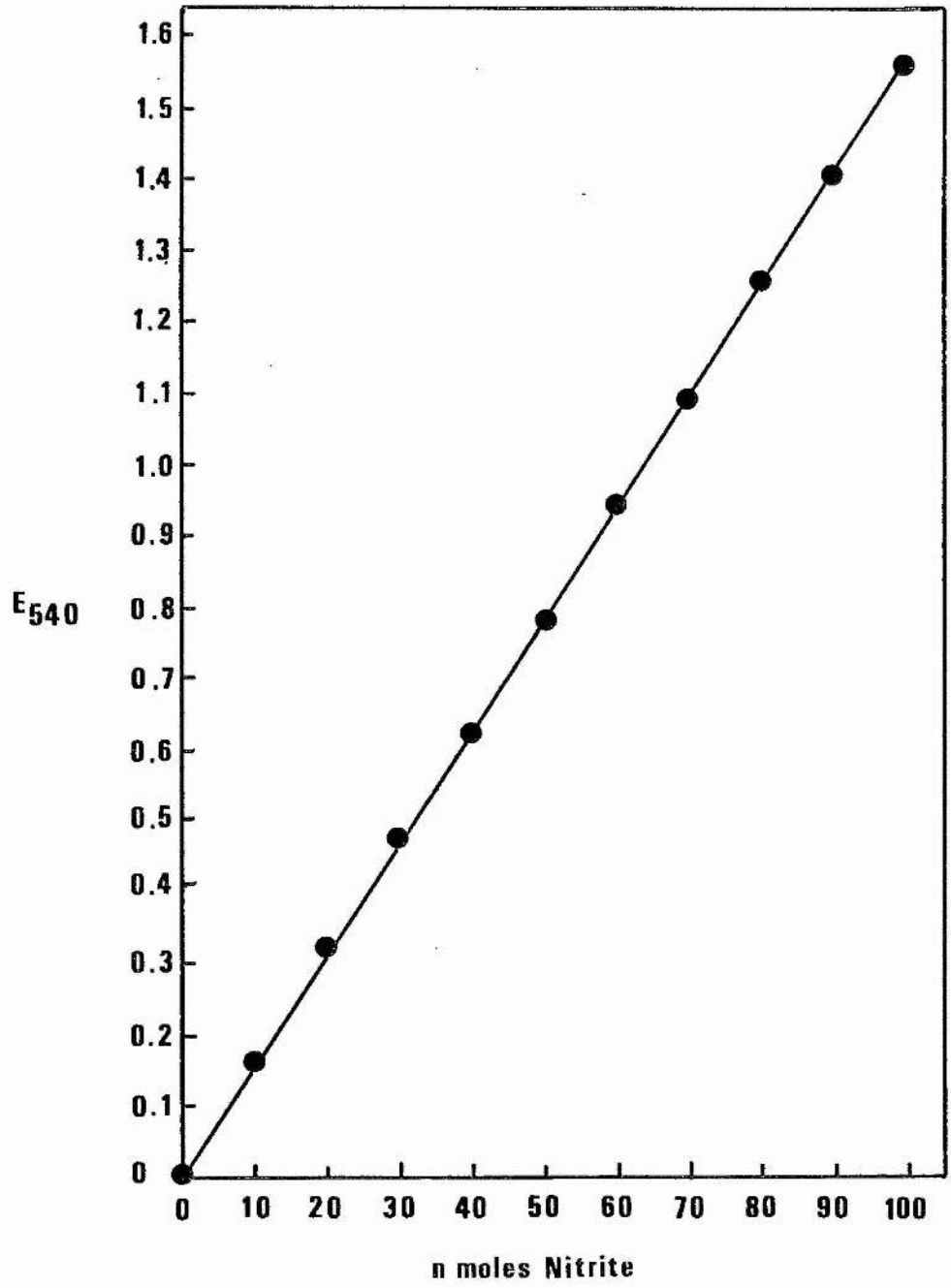
FMNH-Nitrate Reductase

This activity was assayed by a slight modification of the method of Wray and Filner (1970). The assay mixture contained 0.4 ml of 0.1 M potassium phosphate buffer pH 7.5, 0.3 ml of 0.2 mM FMN, 0.1 ml of 0.1 M potassium nitrate and 0.1 ml of enzyme. The reaction was started by the addition of 0.1 ml of 10 mg/ml sodium dithionite in 95 mM sodium bicarbonate which reduces the FMN to its pale yellow form. After incubation at 25°C for an appropriate time period (10 min for crude extract), the reaction was terminated by vigorously mixing the tube contents with a

FIG. 1

Nitrite Standard Curve

This plot shows the increase in absorbance at 540 nm due to increasing nitrite concentrations following addition of the Griess-Ilosvay reagents (see Methods, Section III) to standard nitrite solutions.


E_{540}
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 10 20 30 40 50 60 70 80 90 100
n moles Nitrite

Whirlimixer. This reoxidises the FMNH to its bright yellow form. Nitrite production was then determined by means of the same reagents as for NADH-nitrate reductase activity.

Reduced Methyl Viologen-Nitrate Reductase

For this assay the assay mixture contained 0.6 ml of 0.1 M potassium phosphate buffer pH 7.5, 0.1 ml of 1 mM methyl viologen, 0.1 ml of 0.1 M potassium nitrate and 0.1 ml of enzyme. The reaction was started by the addition of 0.1 ml of 10 mg/ml sodium dithionite in 95 mM sodium bicarbonate which reduces the methyl viologen to its blue form. After incubation of the tube contents at 25°C for a suitable time-period the reaction was terminated by vigorous mixing of the tube contents on a Whirlimixer. This reoxidises the methyl viologen to its colourless form. The nitrite formed during the reaction was then determined in the same way as for NADH-Nitrate reductase.

Reduced Methyl Viologen-Nitrite Reductase

This activity was measured in exactly the same way as reduced methyl viologen-nitrate reductase with the exception that 0.1 ml of 1 mM sodium nitrite replaced the 0.1 ml of 0.1 M potassium nitrate in the reaction mixture.

NADH-Cytochrome c Reductase

This activity was assayed by the method of Wray and Filner (1970). The reaction mixture contained 0.1 ml potassium phosphate buffer pH 7.5, 0.08 ml of 2% (w/v) cytochrome c, 0.02 ml of 0.1 mM NADH, distilled water and enzyme to a total volume of 0.4 ml. The increase in absorbance at 550 nm due to the reduction of cytochrome c was followed after addition of enzyme to start the reaction. In this and all other spectrophotometric assays described, the change in optical density was followed using either (a) a Pye Unicam SP 500 Series II UV/visible spectrophotometer linked to a Pye Unicam AR 25 chart recorder or (b) a Gilford-modified Pye-Unicam SP 500 Series I UV/visible spectrophotometer linked to a Gilford 600 chart recorder.

Changes in absorbance were converted to moles of cytochrome c by means of its molar extinction coefficient of 2.1×10^{4} M^{-1} (Siegel, Davis and Kamin, 1974).

Ferrocyanide-Activated NADH-Cytochrome c Reductase

This activity was first reported by Wallace and Johnson (1978) and was assayed essentially by their method. This assay differs from the NADH-cytochrome c reductase assay in that 0.1 ml of 0.96 mM potassium ferrocyanide replaced 0.1 ml of distilled water in the reaction mixture. The reduction of cytochrome c was again followed by measuring the increase in absorbance at 550 nm. Unlike

the assay for NADH-cytochrome c reductase, this assay was started by the addition of NADH in order that the enzyme and ferrocyanide had had time to mix prior to commencement of the assay.

Catalase

Catalase was assayed by a modification of the method of Beers and Sizer (1952). 0.4 ml of 30-volume hydrogen peroxide was added to 100 ml of 0.1 M potassium phosphate buffer pH 7.5. 3 ml of this solution was then pipetted into a cuvette and the reaction started by the addition of 0.03 ml of enzyme. The decrease in absorbance at 240 nm due to the decrease in hydrogen peroxide concentration was followed by means of a recording spectrophotometer.

Alcohol Dehydrogenase

Alcohol Dehydrogenase was assayed by a modification of the method of Vallee and Hoch (1955). The following reaction mixture was prepared:

0.1 M Tris/HCl buffer pH 8.5	20 ml
3 mg/ml NAD^+	20 ml
2 mg/ml dithiothreitol	13.3 ml
1% (v/v) ethanol	20 ml

3 ml of this mixture was pipetted into a cuvette and the reaction started by the addition of 0.03 ml of enzyme.

The increase in absorbance at 340 nm due to the production of NADH was followed by means of a recording spectrophotometer.

NADH-DCPIP Reductase

This activity was measured essentially by the method of Oji and Izawa (1969). Into a cuvette were pipetted 0.2 ml of 0.1 M potassium phosphate buffer pH 7.5, 0.1 ml of enzyme, and 0.1 ml of 0.66 mM DCPIP. The reaction was started by the addition of 0.1 ml of 0.83 mg/ml NADH and the decrease in absorbance at 620 nm due to the reduction of DCPIP followed by means of a recording spectrophotometer. When activities were very low, the change in absorbance was determined over a 45 minute period without the use of a recording spectrophotometer.

Changes in absorbance were converted to moles of DCPIP reduced by means of the experimentally determined molar extinction coefficient of 1.012×10^4.

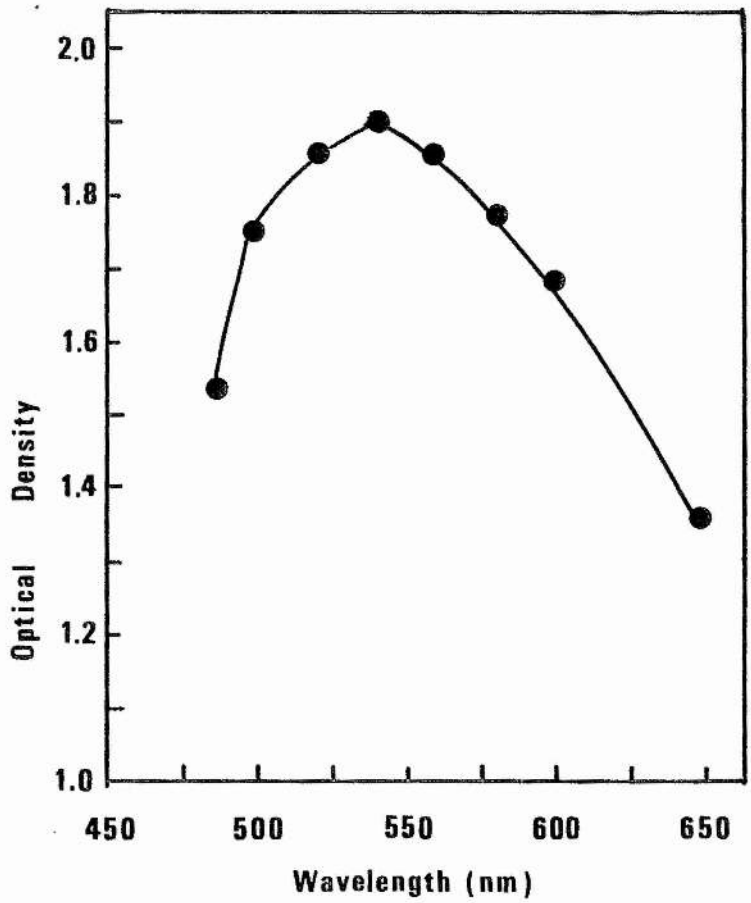
NADH-NBT Reductase

The absorbance maximum of reduced NBT was first determined to be 540 nm (Fig. 2) using dithionite-reduced NBT. The increase in absorbance was then measured for a mixture comprising 0.1 ml of 0.1 M potassium phosphate buffer pH 7.5, 0.1 ml of 0.9 mg/ml NADH, 0.1 ml of 2 mg/ml NBT and 0.1 ml of enzyme. The reaction was started by the

FIG. 2

Partial Absorption Spectrum of Reduced NBT

A 1 mg/ml solution of NBT in 0.1 M phosphate buffer, pH 7.5 was reduced by the dropwise addition of 10 mg/ml sodium dithionite contained in 95 mM sodium bicarbonate until a deep purple colour was obtained. The absorbance of this solution at selected wavelengths was then determined using oxidised NBT in 0.1 M phosphate buffer, pH 7.5 as a blank.


2.0
1.8
1.6
1.4
1.2
1.0
Optical Density
450
500
550
600
650
Wavelength (nm)

addition of enzyme and the reaction followed by means of a recording spectrophotometer. When activities were very low, the change in absorbance was determined over a 45 minute period. Changes in absorbance were converted to moles of NBT reduced by means of the experimentally determined molar extinction coefficient of 5.742×10^3 using dithionite-reduced NBT.

NADH-Ferricyanide Reductase

The decrease in absorbance at 410 nm due to the reduction of ferricyanide was followed in a mixture comprising 0.1 ml of 2.5 mg/ml NADH, 0.1 ml of 4 mM potassium ferricyanide, 0.1 ml of 0.1 M potassium phosphate buffer, pH 7.5 and 0.1 ml of enzyme. The reaction was started by addition of enzyme and followed at 420 nm by means of a recording spectrophotometer. Changes in absorbance were converted to moles of ferricyanide reduced by means of the molar extinction coefficient of 1×10^3 reported by Mahler and Cordes (1971).

Non-Enzymic Proteins

(i) Myoglobin and ferritin were both detected by means of their absorbance at 415 nm.

(ii) Bovine serum albumin and fibrinogen were both detected by means of their absorbance at 280 nm.

SECTION IV - ANALYTICAL PROCEDURES

Preparation of Sucrose Density Gradients

All sucrose solutions were prepared in 0.1 M potassium phosphate buffer pH 7.5 containing 1 mM EDTA and 10 μM FAD. From a stock solution of 20% (w/v) sucrose contained in this buffer, solutions of 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16% and 18% sucrose were prepared by dilution with buffer lacking sucrose. Three 25 ml polypropylene centrifuge tubes (MSE-34411-138) were then washed in a hot EDTA solution, rinsed in distilled water and then dried thoroughly. Failure to treat the tubes in this manner was found to result in inactivation of enzyme samples subsequently centrifuged in the tubes.

With the cleaned centrifuge tubes supported in a vertical position, and starting with the 18% solution, 2 ml of each successive sucrose solution was carefully pipetted into each tube. Tubes containing gradients were then carefully capped with Nescofilm and placed in a cold room at 4°C for 18 hours to allow equilibration.

Sucrose Density Gradient Centrifugation

Samples for analysis by sucrose density gradient centrifugation were first mixed with aliquots of each of three reference proteins, namely catalase (11.3 S), alcohol dehydrogenase (7.4 S) and myoglobin (2.04 S). 0.4 ml of this mixture was then carefully layered on top of each of

the gradients, which were then placed inside the buckets of a 3×23 ml swing out rotor. The tube assemblies were then balanced by the dropwise addition of water to the buckets, between the centrifuge tube and the bucket wall. The rotor was then assembled and placed in an MSE Superspeed 65 Preparative Ultracentrifuge (Mark I or Mark II) and centrifuged for 24 hours at 94 000 g and 3°C.

Fractionation of Sucrose Density Gradients

Following centrifugation, the gradients were carefully removed from the rotor and fractionated from the bottom, by means of an MSE Tube Piercer, into 41 fractions of 15 drops each. Fractions were then assayed for the required enzyme activities. Gradient linearity was confirmed by means of a Bellingham and Stanley Abbé-type refractometer. (A program for converting the reading from this instrument into Refractive Index by means of a Texas TI-58/59 programmable calculator is presented in Appendix I.)

Calculation of Sedimentation Coefficients

Sedimentation coefficients were determined by reference to the sedimentation characteristics of the three reference proteins included in each gradient. The distribution of catalase, alcohol dehydrogenase and myoglobin in fractions from the gradient was determined and these were plotted against fraction number. The peak fraction

numbers of each of the reference proteins were then plotted against their known sedimentation coefficients to yield a straight line and gradients where this plot deviated significantly from a straight line were discarded. The sedimentation coefficient(s) of the protein(s) under study could then be read off this plot from a knowledge of their peak fraction numbers.

This procedure of using three internal standards was found to give much more reproducible results than the more commonly used Martin and Ames (1961) technique which relies on reference to only one protein.

Sephadex G200 Gel Filtration

Samples for analysis by Sephadex G200 gel filtration were first mixed with aliquots of three reference proteins, namely catalase (5.2 nm), alcohol dehydrogenase (4.6 nm) and bovine serum albumin (3.5 nm). 1 ml of this mixture was then applied to a column (2 cm × 52 cm) of Sephadex G200 previously equilibrated in Buffer II. The enzyme was then eluted with Buffer II and fractions collected with either a Copley Fractomin fraction collector or an LKB Ultrorac fraction collector. Flow rates were maintained at about 12 ml per hour and 2 ml fractions were collected. All column operations were carried out at 4°C.

Calculation of Stokes Radii

Stokes radii were determined by reference to the

elution characteristics of the three reference proteins. The elution volume corresponding to the peak of each of these proteins can be related to the Stokes radius by means of the correlation of Porath (1963) which uses the equation

$$K_d = \frac{V_e - V_o}{V_t - V_g - V_o}$$

where V_e is the elution volume; V_o is the void volume of the column (determined separately using Blue Dextran); V_t is the total volume of the column and V_g is the volume not accessible to solvent which can be defined as B×d where B is the bed volume per gram of dry Sephadex G200 (= 30 ml) and d is the density of dry Sephadex G200 (= 1.65 cm^3/g).

A plot of $K_d^{1/3}$ against Stokes radius for the reference proteins should yield a straight line. A calibration plot showing additional reference proteins is presented in Fig. 3 to show that the plot is linear outwith the limits of the reference proteins used routinely.

Thus, by determining the elution volume of the protein under study, the value of $K_d^{1/3}$ can be determined and converted to Stokes radius by means of the calibration plot compiled from the reference proteins eluted from the same column. This technique thus prevents errors arising due to changes in column characteristics between runs.

FIG. 3

Calibration Plot for Sephadex G200 Gel Filtration

This plot shows the linear relationship between Stokes radius and $K_d^{1/3}$ for a range of proteins of known Stokes radius. These are:

a. Myoglobin (2 nm)

b. Bovine Serum Albumin (3.5 nm)

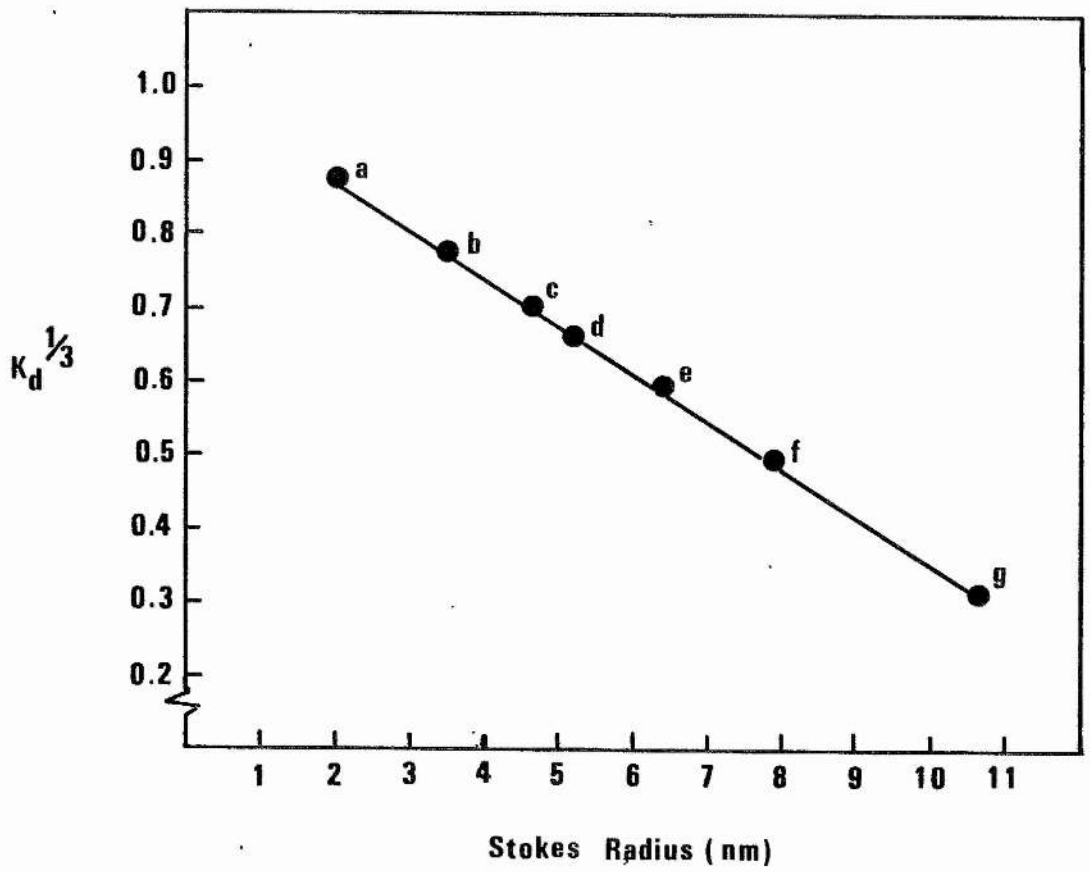
c. Yeast Alcohol Dehydrogenase (4.6 nm)

d. Catalase (5.2 nm)

e. Urease (6.4 nm)

f. Ferritin (7.9 nm)

g. Fibrinogen (10.7 nm)


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
$K_d^{1/3}$
a
b
c
d
e
f
g
1
2
3
4
5
6
7
8
9
10
11
Stokes Radius (nm)

(A program for converting peak fraction number into elution volume and $K_d^{1/3}$ by means of a Texas TI-58/59 programmable calculator is presented in Appendix II. An additional programme, for use with gels where the values of B and d are not known, is presented in Appendix III.)

Calculation of Molecular Weights, Frictional Ratios and Axial Ratios

The molecular weight of a protein can be determined from a knowledge of its sedimentation coefficient, s, and its Stokes radius, a, by means of the equation

$$M = \frac{6\pi\eta Nas}{(1-\bar{v}\rho)}$$

where η is the viscosity of the medium, (assumed to be 1), N is Avagadro's number ($= 6.02\times10^{23}$), a is the Stokes radius in metres, s is the sedimentation coefficient in sec^{-1}, $\bar{v}$ is the partial specific volume (assumed to be 0.725 cm^3/g) and ρ is the density of the medium (assumed to be 1).

Frictional ratios were calculated by means of the equation

$$\frac{f}{f_o} = \left(\frac{3\bar{v}M}{4\pi N}\right)^{1/3}$$

Frictional ratios are usually found to be within the range 1 to 2, with values close to unity indicating globular

proteins and larger values indicating increasing asymmetry (Siegel and Monty, 1966).

Axial ratios (r_1/r_2) were calculated from frictional ratios using the data presented by Oncley (1941) - assuming a hydration level of 0.1 g H_2O per gram of protein (A. Serafini-Fracassini, personal communication).

(A program for calculating molecular weights and frictional ratios from any values of sedimentation coefficient and Stokes radius by means of a Texas TI-58/59 programmable calculator is presented in Appendix IV.)

SECTION V - ELECTROPHORETIC TECHNIQUES

Preparation of Polyacrylamide Gels

A solution containing 5% (w/v) acrylamide and 0.4% (w/v) methylene bis acrylamide in 0.2 M Tris/HCl pH 8.5 was prepared. 20 ml of this solution was deaerated and then 0.05 ml of TEMED was added followed by 5 mg of ammonium persulphate. This solution was then pipetted into vertically held glass tubes (75 mm × 5 mm) and a few drops of distilled water carefully added to the top of each to ensure a flat top to each gel. The gels were then placed in strong light to facilitate polymerisation, which usually occurred within 20 minutes.

After removal of the distilled water, the gels were placed in a Shandon apparatus for disc gel electrophoresis.

The reservoirs were then filled with 0.08 M Tris/HCl buffer pH 8.5 and the gels preequilibrated at 1-2 mA per tube at 4°C for 20 min before application of the sample. The positive electrode was always connected to the lower reservoir.

Polyacrylamide Gel Electrophoresis

0.5 ml of enzyme sample was first mixed in a test tube with one drop of glycerol and one drop of 0.05% (w/v) methylene blue. After mixing, 50 µl of solution was then applied to each gel and electrophoresis carried out at 4°C and 2.5-3 mA per tube until the methylene blue tracking dye had travelled to within 1 cm of the bottom of the tubes. This usually took 4-5 hours.

Staining of Gels

(i) Protein Stain

Gels were removed from the tubes and placed in test tubes which were then filled with a solution of Coomassie Brilliant Blue R250 prepared as follows:

1.25 g	Coomassie Brilliant Blue R250
227 ml	Methanol
227 ml	Distilled Water
46 ml	Glacial Acetic Acid

Gels were stained for between 2 and 6 hours depending

on the sample applied. After this period the gels were transferred to a destaining solution in a test tube. The destaining solution consisted of

250 ml	Methanol
75 ml	Glacial Acetic Acid
675 ml	Distilled Water

The destaining solution was changed regularly until the process was completed.

(ii) Reduced Methyl Viologen Nitrate Reductase Activity Stain

This activity stain is based on a modification of the method used by Hucklesby and Hageman (1973) for nitrite reductase. For this stain the gels were placed in test tubes to which were added 7 ml of 0.1 M potassium phosphate buffer pH 7.5, 1 ml of 0.1 M potassium nitrate, 1 ml of 5 mM methyl viologen and 0.5 ml of a 10 mg/ml solution of sodium dithionite in 95 mM sodium hydrogen carbonate.

The dithionite solution was added last to reduce the methyl viologen to its blue-coloured form. The test tubes were sealed with Nescofilm and the tube contents mixed by inversion. The blue solution stains the gel blue except where reduced methyl viologen nitrate reductase activity oxidises the blue methyl viologen to its leuco form. Thus an achromic band appears at the site of nitrate reductase activity. Immediately after the appearance of

this band, the gels are transferred to a solution of 2.5 % (w/v) triphenyl-tetrazolium chloride which makes the activity stain permanent by reacting with the reduced (blue) methyl viologen to produce a red formazan derivative. The colourless band of nitrate reductase activity remains colourless and is clearly visible against the red background. This solution must be protected from strong light to prevent precipitation of the formazan dye.

(iii) NADH-Dehydrogenase Activity Stain

Nitroblue-tetrazolium was used to detect NADH-dehydrogenase activity on gels. After removal from the electrophoresis tubes the gels were placed in test-tubes to which was added a solution containing 5 mg of nitroblue tetrazolium and 5 mg of NADH in 25 ml of 0.1 M potassium phosphate buffer pH 7.5. This was first used by Solomonson (1975) to detect NADH dehydrogenase activity in column fractions. The tubes were then wrapped in foil as exposure to light was found to cause precipitation in the staining solution. NADH-dehydrogenase activity was detected by the appearance of purple-black bands in the gel. Gels stained in this way can be kept indefinitely in the staining solution provided that darkness is maintained.

Preparation of Samples for SDS Gel Electrophoresis

Samples of reference proteins were prepared for SDS

gel electrophoresis by means of a slight modification of the method of Weber and Osborn (1969) in which samples were incubated for 3 hours in 0.02 M sodium phosphate buffer, pH 7.0, containing 2% (w/v) SDS and 1% (v/v) 2-mercapto-ethanol at 37°C. Nitrate reductase samples in 20 mM potassium phosphate buffer, pH 7.5 containing 10 µM FAD, 0.1 mM EDTA and 1 mM cysteine were mixed with an equal volume of 0.02 M sodium phosphate buffer pH 7.0 containing 2% (w/v) SDS and kept at room temperature for 15 hours prior to addition of 2-mercaptoethanol to a final concentration of 1% (v/v) and then incubated at 37°C for 3 hours.

Prior to application to the gels, 100 µl of each sample was mixed with 10 µl of 0.05% (w/v) bromophenol blue tracking dye, 10 µl of 2-mercaptoethanol and one drop of glycerol. A 50 µl aliquot of the mixture was applied to the gels.

Preparation of Gels for SDS Gel Electrophoresis

Gel buffer contained 2.6 g $NaH_2PO_4.H_2O$, 12.866 g of $Na_2HPO_4.7H_2O$ and 2 g of SDS per litre. This buffer concentration is one third of that used by Weber and Osborn (1969), this modification allowing electrophoresis to be completed in a much shorter time.

15 ml of this gel buffer was mixed with 13.5 ml of a 22.2 % (w/v) acrylamide solution containing 0.6% (w/v) methylene bis acrylamide. After deaeration for 5 min,

1.5 ml of freshly prepared ammonium persulphate solution (10 mg/ml) and 0.04 ml of TEMED were added, and the solution pipetted into each of eight electrophoresis tubes (98 mm × 5 mm). A few drops of distilled water were carefully placed on top of each gel to ensure a flat top and the tubes placed in strong light for 30 min during which time the gels polymerised.

SDS Gel Electrophoresis

After polymerisation, the water layer was removed from the gels and the tubes placed in the electrophoresis apparatus. The reservoirs were then filled with a 1:1 dilution of gel buffer with distilled water and the gels preequilibrated at 1 mA per tube for 15 min at room temperature, with the positive electrode connected to the lower reservoir. Protein samples (50 µl) were then applied to the gels and electrophoresis carried out at 3 mA per tube at room temperature. The blue tracking dye migrates to within 1 cm of the bottom on the tubes within 4-5 hours under these conditions.

After electrophoresis, the gels were removed from the above tubes and the length of gel and the distance moved by the tracking dye then measured for each gel. The gels were then placed in test tubes containing Coomassie Brilliant Blue staining solution (as described previously) for approximately six hours after which the gels were transferred to test-tubes containing destaining solution (as described previously). This solution was

changed regularly until the gels had completely destained at which times the length of each gel was again measured, together with the distance moved by each of the protein bands visible on the gels. Gels were then scanned using a Vitatron densitometer.

Calculation of Molecular Weights Following SDS Gel Electrophoresis

Making the assumption that all the gels swell evenly during staining and destaining, the mobility of each of the protein bands can be calculated by means of the equation reported by Weber and Osborn (1969):

$$\text{Mobility}=\frac{\text{distance of protein migration}}{\text{length after destaining}}\times\frac{\text{length before destaining}}{\text{distance of dye migration}}$$

Thus calculated, the mobilities are then plotted against the known molecular weights of the reference proteins using a semi-logarithmic plot. A calibration plot compiled in this way can then be used to determine the molecular weights of proteins present in the nitrate reductase samples which had been run in parallel with the standards.

SECTION VI - PREPARATION OF BLUE DEXTRAN SEPHAROSE

Binding of Blue Dextran 2000 to Sepharose 4B

The method used for the binding of Blue Dextran 2000 to Sepharose 4B was essentially that of Sherrard and

Dalling (1979), the only difference being that CNBr-activated Sepharose 4B was purchased from Pharmacia instead of being synthesised. 3 g of CNBr-activated Sepharose 4B was swollen in 200 ml of ice-cold 1 mM HCl and washed on a sinter with excess (500 ml) 1 mM HCl to remove preservatives. To 10 ml of this swollen gel was added 20 ml of a solution containing 0.4 g of Blue Dextran 2000 in 0.4 M Na_2CO_3 pH 8.0 and the mixture placed in a screw-top vial which was then mixed at 4°C for 18 hours using an end-over-end tumbler. Following this the gel was washed on a sinter with distilled water and then suspended in 0.1 M Tris/HCl pH 8.0 for 2 hours at 4°C to block unreacted sites on the Sepharose. The gel was then washed with five cycles of two buffers differing in pH, namely 0.1 M sodium citrate buffer pH 4.0 and 0.1 M borate buffer pH 8.0. 50 ml of each buffer was used in each cycle for every 10 ml of swollen gel.

Determination of Extent of Binding of Blue Dextran 2000 to CNBr-Activated Sepharose 4B

0.4 g of Blue Dextran 2000 was dissolved in 20 ml of 0.1 M Na_2CO_3 pH 8.0, as for the coupling experiment. This was then diluted to 1 litre with distilled water and the optical density determined at 600 nm. Following a coupling experiment, all the washings of the gel were collected, diluted to 1 litre with distilled water, and the optical density at 600 nm again determined.

For 10 ml of swollen gel, the extent of dye binding to the gel can be determined from the equation:

$$\text{Amount of dye bound per ml of gel} = \frac{\left(400 \times \frac{E_{600}\ \text{washings}}{E_{600}\ \text{initial}}\right)}{10}$$

The highest extent of binding achieved was 11.7 mg blue dextran bound per ml of swollen gel which compares favourably with the value of 13 mg/ml reported by Sherrard and Dalling (1979).

SECTION VII - PROTEIN ESTIMATIONS

Protein concentrations were determined by the method of Lowry *et al.* (1951). Aliquots of sample were first precipitated with at least an equal volume of 10% TCA. The samples were then left overnight to allow complete precipitation following which they were washed with 2 lots of 3 ml of 95% ethanol, the precipitate being collected on each occasion by centrifugation at top speed in a bench-top centrifuge for 5 min. Each sample was then washed with 3 ml of diethyl ether and after collection of the precipitated protein by centrifugation they were evaporated to dryness under a stream of nitrogen.

The dried protein samples were then dissolved in 1 M NaOH and 0.2 ml of this solution taken for protein determination. All determinations were done in duplicate. The following solutions were required:

(1) Reagent A: 2% Na_2CO_3 in 0.1 M NaOH

(2) Reagent B: prepared freshly by mixing equal volumes of 1% (w/v) copper sulphate and 2% (w/v) sodium tartrate.

(3) Reagent C: prepared from Reagents A and B by mixing in the ratio 50:1.

Each 0.2 ml protein sample in 1 M NaOH was then mixed with 1 ml of Reagent C and the solution left to stand for at least 15 min. 0.1 ml of Folin-Ciocalteu Phenol Reagent was then added to each with vigorous mixing and the solutions were then left to stand for 1 hour at room temperature after which the absorbance at 500 nm was determined against a reagent blank.

Protein standards, ranging from 0 to 100 µg BSA in 1 M NaOH were performed at the same time and used to form a calibration plot from which the amounts of protein in the samples were determined.

RESULTS

CHAPTER 1

PURIFICATION OF BARLEY NITRATE REDUCTASE

INTRODUCTION

Nitrate reductase is a large and complex enzyme. As indicated in the main Introduction, there are both flavin and cytochrome components of the enzyme, and Mo is required for its activity. However, very little information is available about the number of moles of these present or the subunit composition of higher plant nitrate reductase. This situation has arisen because of difficulties encountered during the purification of this enzyme resulting in low yields and impure preparations with which definitive characterisations of the enzyme were not possible.

In 1975, however, Solomonson reported a method for the purification of *Chlorella* nitrate reductase utilising affinity chromatography through Blue Dextran - Sepharose. It had previously been observed that some proteins, e.g. pyruvate kinase (Blume *et al.*, 1971) and glutathione reductase (Staal, Visser and Veeger, 1969) behaved anomalously when subjected to gel filtration in the presence of Blue Dextran. Instead of being retarded by the gel, these proteins were eluted with the Blue Dextran at the void volume of the column. This behaviour was subsequently exploited by the preparation of Blue Dextran - Sepharose affinity columns for use during the final stages of purification of these enzymes (Ryan and Vestling, 1974).

It was proposed (Thompson, Cass and Stellwagen, 1975) that Blue Dextran complexed with a wide range of

proteins because of a specific interaction with a super-secondary structure called the dinucleotide fold which involves about 120 amino acids arranged in a β-sheet core composed of five or six parallel strands connected by α-helical loops (Rossman, Moras and Olsen, 1974). This dinucleotide fold was known to form the NAD-binding site for lactate, malate and glyceraldehyde phosphate dehydrogenases, the ATP binding site in phosphoglycerate kinase and to be present in the structures of alcohol dehydrogenase, adenylate kinase and phosphoglycerate mutase. Thompson, Cass and Stellwagen (1975) demonstrated binding of each of these enzymes to Blue Dextran-Sepharose and each could be eluted by low concentrations of their nucleotide substrates.

Subsequently, Solomonson (1975) demonstrated the 640-fold purification of *Chlorella* nitrate reductase using Blue Dextran-Sepharose to yield electrophoretically homogeneous enzyme with an overall yield of 60%. The whole purification took one day, compared to the ten days necessary using more conventional techniques (Solomonson *et al.*, 1975). The rapid purification of *Chlorella* nitrate reductase (Solomonson, 1975) utilised only three steps, namely protamine sulphate treatment, $(NH_4)_2SO_4$ precipitation and Blue Dextran-Sepharose affinity chromatography from which nitrate reductase activity was eluted by NADH concentrations as low as 5 μM.

It was therefore decided to attempt to purify barley

nitrate reductase by this method. However, just prior to commencement of the work, Pharmacia marketed their version of Blue Dextran-Sepharose, called Blue-Sepharose CL-6B. Unlike Blue Dextran-Sepharose, commercial Blue-Sepharose has the same chromophore (Cibacron Blue F3GA) bound directly to the Sepharose matrix (see Fig. 4), omitting the dextran spacer molecule. Believing that commercial Blue-Sepharose would operate in the same way as Blue Dextran-Sepharose it was decided to use commercial Blue-Sepharose for the purification of barley nitrate reductase.

RESULTS

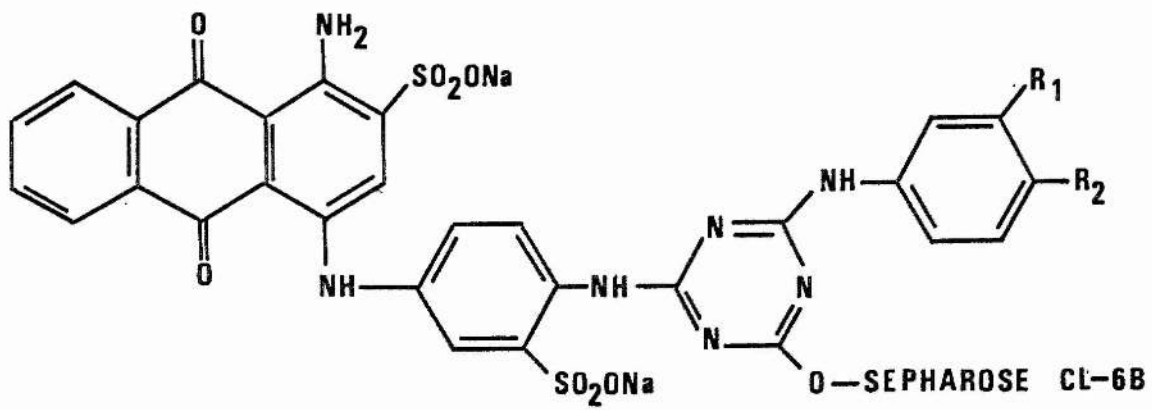
SECTION I - CHOICE OF EXTRACTION BUFFER

In previous work Wray and Filner (1970) used 0.1 M Tris/HCl buffer, pH 7.5 containing 1 mM cysteine to extract barley nitrate reductase. Cysteine was present to protect sulphydryl groups on the enzyme from both phenolic oxidation and disulphide bond formation. However, as Solomonson (1975) used a phosphate buffer during Blue Dextran-Sepharose chromatography and because of the known stimulatory effect of phosphate on nitrate reductase activity (Sanderson and Cocking, 1964; Ferrari and Varner, 1970) it was decided to compare the extraction buffer of Wray and Filner (1970) with one based on that of Solomonson (1975). FAD was also included in the extraction buffer because of its known stabilising effect on nitrate reductase (Wray and Filner, 1970; Zumft *et al.*, 1970) and EDTA

FIG. 4

Structure of Cibacron Blue F3GA

The structure of Cibacron Blue F3GA is shown opposite together with the site of linkage to Separose CL-6B to form Blue-Sepharose. In Blue Dextran-Sepharose a dextran spacer molecule occurs between the dye and the Sepharose matrix.



R_1 = H or SO_2ONa

R_2 = SO_2ONa or H

was included to prevent any heavy metal ions from complexing with the enzyme. A modification of the extraction buffer of Wray and Filner (1970), in which the level of sulphydryl protection was increased by the replacemnt of 1 mM cysteine with 10 mM mercaptoethanol, was also tested.

2 g samples of barley shoots were ground in a chilled mortar and pestle with 6 ml of each buffer and cell debris removed by centrifugation at 38 000 g for 20 min. Nitrate reductase activity was then determined in each of the supernatants as indicated below in Table 2.

TABLE 2

Buffer Composition	Nitrate Reductase Activity (nmoles nitrite formed per min/g shoots)
0.1 M Tris/HCl, pH 7.5, 1 mM cysteine	60
0.1 M Tris/HCl, pH 7.5, 10 mM mercaptoethanol	42
0.05 M potassium phosphate, pH 7.5, 0.1 mM EDTA, 1 mM cysteine, 10 µM FAD	75

Since extraction with the phosphate buffer yielded the most nitrate reductase activity it was decided to continue with this buffer (Buffer I) throughout the course of the work.

SECTION II - AFFINITY CHROMATOGRAPHY WITH BLUE-SEPHAROSE

Method of Solomonson (1975)

The method reported by Solomonson (1975) for the purification of *Chlorella* nitrate reductase involved only three steps, namely protamine sulphate treatment, $(NH_4)_2SO_4$ fractionation and Blue Dextran-Sepharose affinity chromatography. For this final step, enzyme in extraction buffer was applied to the column which was then washed with a column volume of extraction buffer to remove unabsorbed protein. The column was then washed with 2-3 column volumes of 0.4 M potassium phosphate buffer and the high ionic strength of this removed many other proteins from the column, but not nitrate reductase. After reequlibration of the column with extraction buffer, nitrate reductase activity was eluted by the application of a linear gradient of 0-100 µM NADH contained in extraction buffer.

When attempting to repeat this method of Solomonson (1975) with barley nitrate reductase it was found that protamine sulphate treatment resulted in the loss of over 50% of the nitrate reductase activity and so this step was omitted in subsequent experiments.

40 g of barley shoots were therefore harvested and ground in a chilled mortar and pestle with cold Buffer I (3 ml buffer per gram shoots) and cell debris removed by centrifugation at 38 000 g for 20 min. Nitrate reductase was precipitated from the resulting supernatant by its

adjustment to 60% saturation with $(NH_4)_2SO_4$ as described in Methods, Section II, and dissolved in 5 ml of Buffer I. This was then applied to a column (1 cm × 9 cm) of Blue-Sepharose previously equilibrated in Buffer I and the column washed with Buffer I. All the nitrate reductase activity bound to the column which was then washed with 0.4 M potassium phosphate buffer pH 7.5, containing 0.1 mM EDTA, 10 μM FAD and 1 mM cysteine, in order to remove more loosely-absorbed protein from the column. However, in contrast to the results of Solomonson (1975) it was found that this treatment resulted in elution of 19.5% of the applied barley nitrate reductase activity (Fig. 5). In further contrast to the results of Solomonson (1975) it was also found that, following reequilibration of the column with Buffer I, application of a linear gradient of 0-100 μM NADH failed to result in the elution of any nitrate reductase activity (Fig. 5).

Thus the results reported here for barley nitrate reductase are in conflict with those of Solomonson (1975) with *Chlorella* nitrate reductase. This could either be due to differences between the nitrate reductases from the two sources or, alternatively, to the use in the work reported here of commercially-available Blue-Sepharose rather than Blue Dextran-Sepharose. This matter was apparently resolved by the publication of a paper reporting the purification of spinach nitrate reductase (Notton, Fido and Hewitt, 1977). These authors obtained identical results to those reported here, but instead used Blue

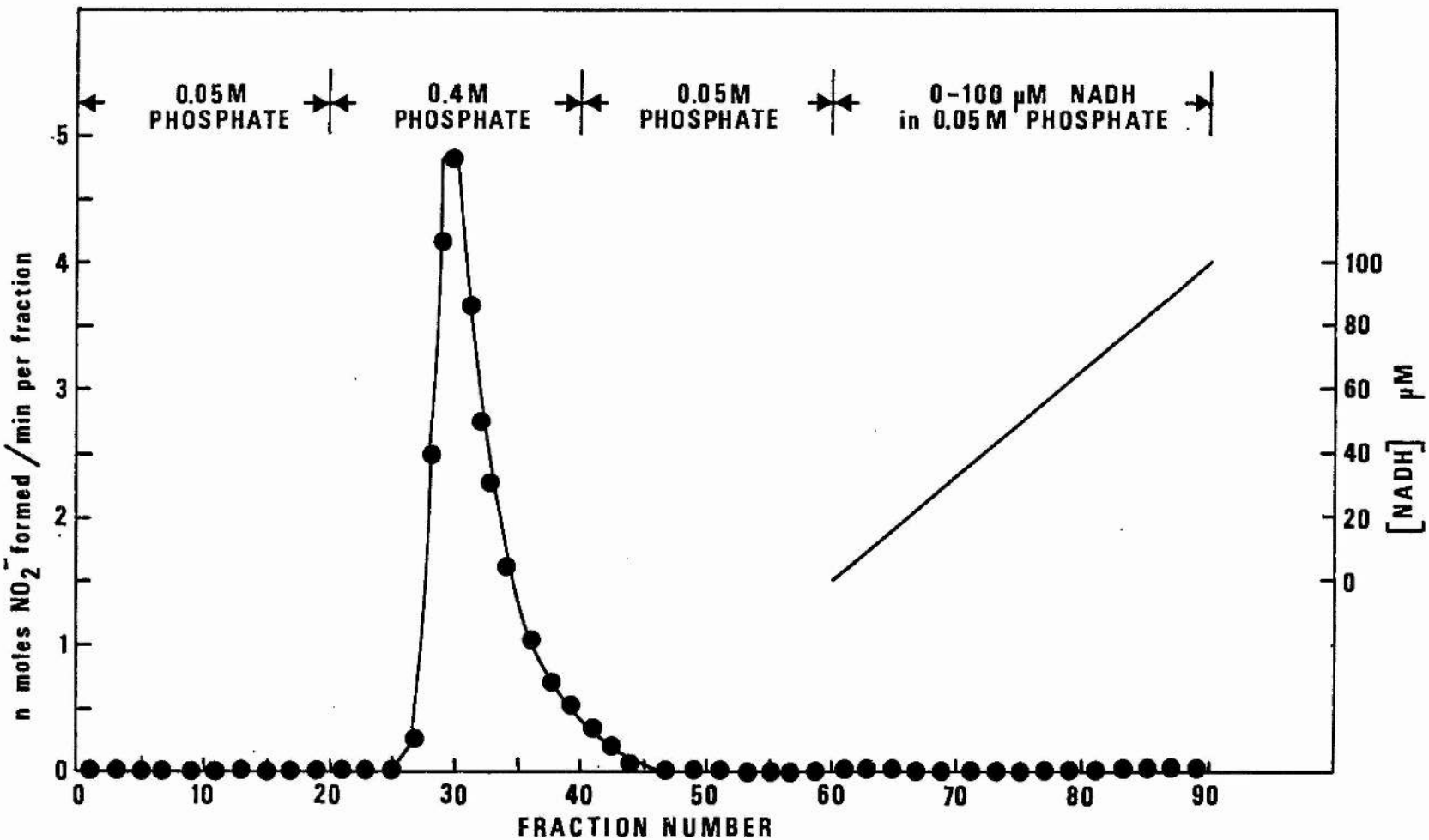

0.05M PHOSPHATE
0.4M PHOSPHATE
0.05M PHOSPHATE
0-100 µM NADH in 0.05M PHOSPHATE
n moles NO_2^- formed /min per fraction
[NADH] µM
FRACTION NUMBER
0
1
2
3
4
5
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80
90

FIG. 5

Effect of Eluting Blue-Sepharose with 0.4 M Phosphate Buffer

This figure demonstrates the ability of 0.4 M phosphate buffer to elute barley nitrate reductase activity from a column (1 cm × 9 cm) of Blue-Sepharose. No further activity could be eluted by a linear gradient of 0 - 100 μM NADH in Buffer I applied to the column following elution with 0.4 M phosphate buffer.

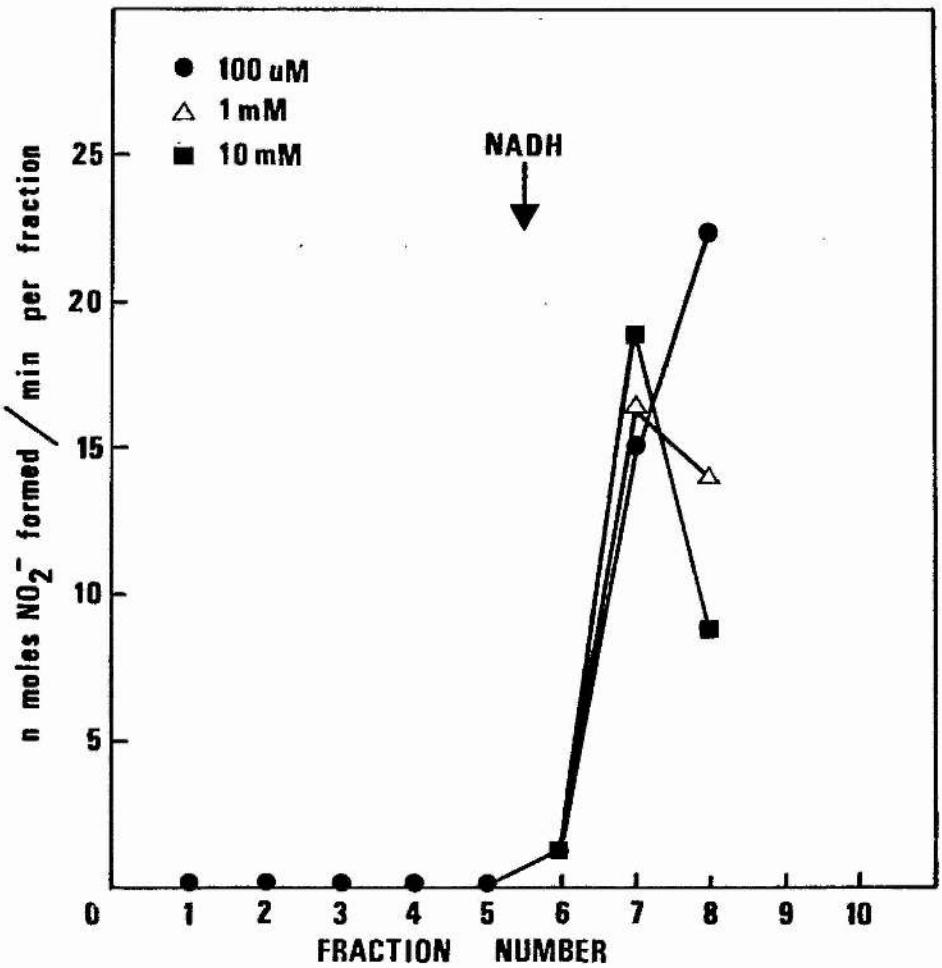

100 uM
1 mM
10 mM
NADH
n moles NO_2^- formed / min per fraction
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
FRACTION NUMBER

FIG. 6

Elution of Nitrate Reductase from Blue-Sepharose by NADH

This figure shows the ability of NADH to elute nitrate reductase from Blue-Sepharose following omission of the preliminary elution with 0.4 M phosphate buffer. All NADH concentrations were effective but the highest activities were recovered following elution with the lowest concentration of NADH.

Dextran-Sepharose, indicating that the deviation from the results of Solomonson (1975) is due to a difference between the nitrate reductases from *Chlorella* and higher plants rather than to the use of differing affinity media.

Direct Elution of Nitrate Reductase by NADH

The previous results (Fig. 5) indicated that nitrate reductase could not be eluted from Blue-Sepharose by a linear gradient of 0-100 μM NADH. It was decided, therefore, to examine the ability of a range of NADH concentrations to elute nitrate reductase to test the possibility that insufficient NADH was applied in the above experiments.

5 g of barley shoots were harvested, extracted as described in Methods, Section II, and 2 ml of the resulting supernatant applied to each of three columns (0.8 cm × 5 cm) of Blue-Sepharose previously equilibrated in Buffer I. After washing with Buffer I, the columns were eluted with 100 μM, 1 mM and 10 mM NADH, respectively (Fig. 6), omitting the 0.4 M buffer wash used by Solomonson (1975). It is evident (Fig. 6) that all three concentrations of NADH eluted significant levels of nitrate reductase activity. As the major deviation between the method of this experiment and that reported in Fig. 5 is the omission of the 0.4 M buffer wash, it would appear that this wash in some way prevents nitrate reductase activity from being subsequently eluted by NADH.

In view of these results, a further attempt was made to purify barley nitrate reductase utilising this modification of the method of Solomonson (1975). 80 g of barley shoots were harvested and extracted as described in Methods, Section II, and the protein precipitated by 60% $(NH_4)_2SO_4$ collected by centrifugation, dissolved in 10 ml of Buffer I and applied to a column (2 cm × 17 cm) of Blue Sepharose previously equilibrated in Buffer I. The column was then washed with a further 300 ml of Buffer I and nitrate reductase activity eluted by a 150 ml linear gradient of 0-100 μM NADH contained in Buffer I (Fig. 7). Nitrate reductase activity was not, however, eluted as a sharp peak and the yield was only 15% of that applied to the column.

A more discrete peak of nitrate reductase activity was obtained in subsequent experiments where the linear gradient of NADH was replaced by a 'step' elution with 100 μM NADH contained in Buffer I (Fig. 8). This change did not, however, alter the yield of active enzyme which in successive experiments was 14%, 13.5% and 14.5%. It appeared likely that these low yields were a consequence of the prolonged washing of the columns to remove non-absorbed protein prior to the elution of nitrate reductase. Thus, either the removal of protein from the column resulted in decreased stability of nitrate reductase leading to low yields or, alternatively, the inherent instability of nitrate reductase was being compounded by

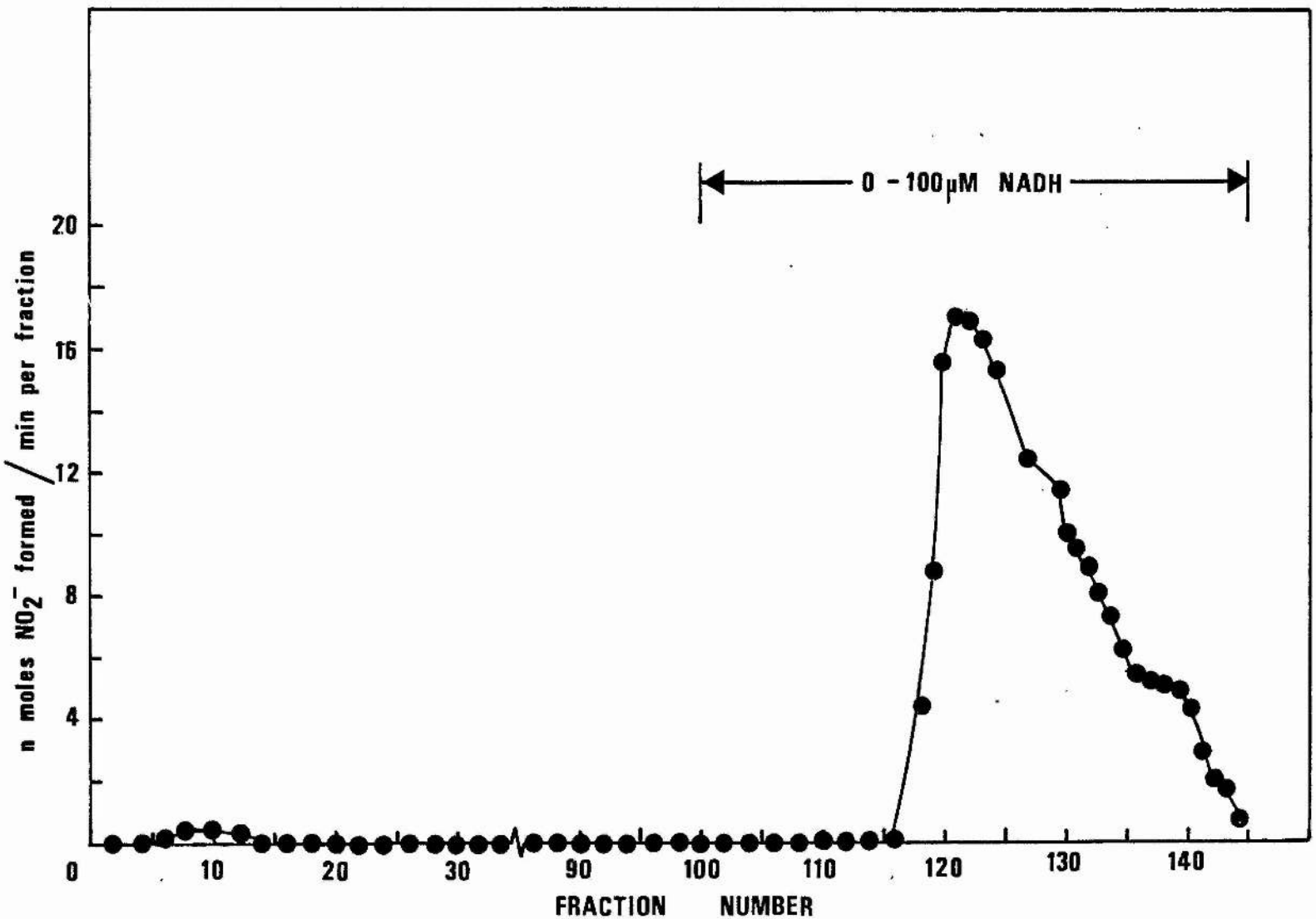

0 - 100μM NADH
n moles NO_2^- formed / min per fraction
0
4
8
12
16
20
10
20
30
90
100
110
120
130
140
FRACTION NUMBER

FIG. 7

Elution of Nitrate Reductase from Blue Sepharose by a Gradient of NADH

This figure shows the elution of nitrate reductase from a column (2 cm × 17 cm) of Blue-Sepharose by a linear gradient of 0 - 100 μM NADH contained in Buffer I. 3 ml fractions were collected throughout.

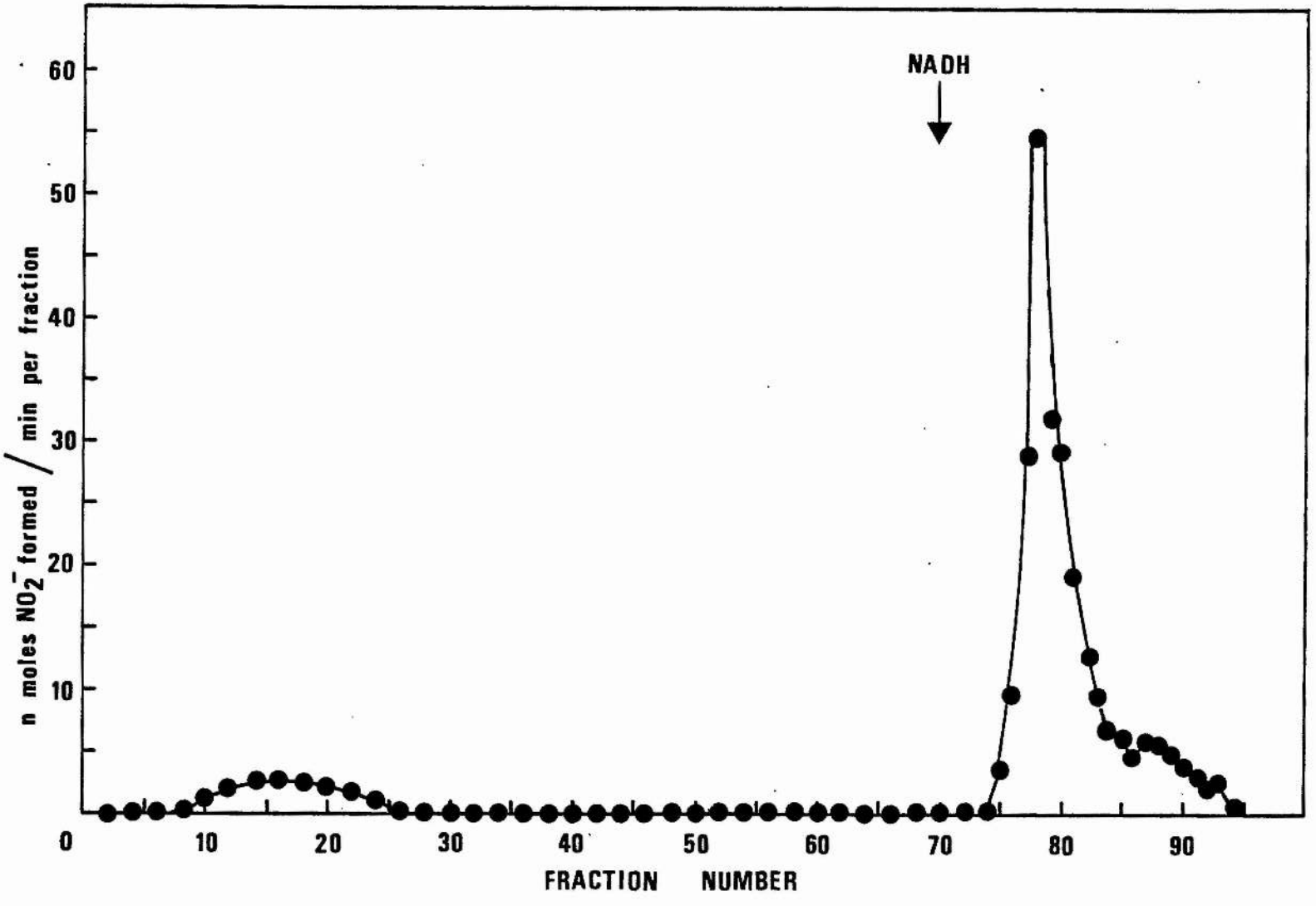

NADH
n moles NO_2^- formed / min per fraction
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

FIG. 8

Elution of Nitrate Reductase from Blue Sepharose with 100 µM NADH

This figure shows the elution of nitrate reductase from a column (2 cm × 8 cm) of Blue-Sepharose with 100 µM NADH contained in Buffer I. 3.2 ml fractions were collected throughout.

the duration of the experiment to result in low yields.

Stabilisation of Nitrate Reductase During Affinity Chromatography

(a) Inclusion of Bovine Serum Albumin

In an effort to increase the yield of nitrate reductase during Blue-Sepharose chromatography, some attempts were made to stabilise the enzyme during the prolonged washing stages. The first method tried was the inclusion of some bovine serum albumin (BSA) in the sample for affinity chromatography. BSA was known to bind to Blue-Sepharose (from the Pharmacia booklet - Blue Sepharose CL-6B) and it was found to increase the yield of nitrate reductase eluted by 100 μM NADH to 20%, presumably by increasing the protein stabilisation during the course of the experiment.

It was discovered, however, that 100 μM NADH could elute BSA from Blue-Sepharose (Fig. 9) resulting in a marked decrease in the specific activity of nitrate reductase, which is also eluted by 100 μM NADH. Thus the usefulness of this method of stabilisation of nitrate reductase, during Blue-Sepharose chromatography, is rather limited.

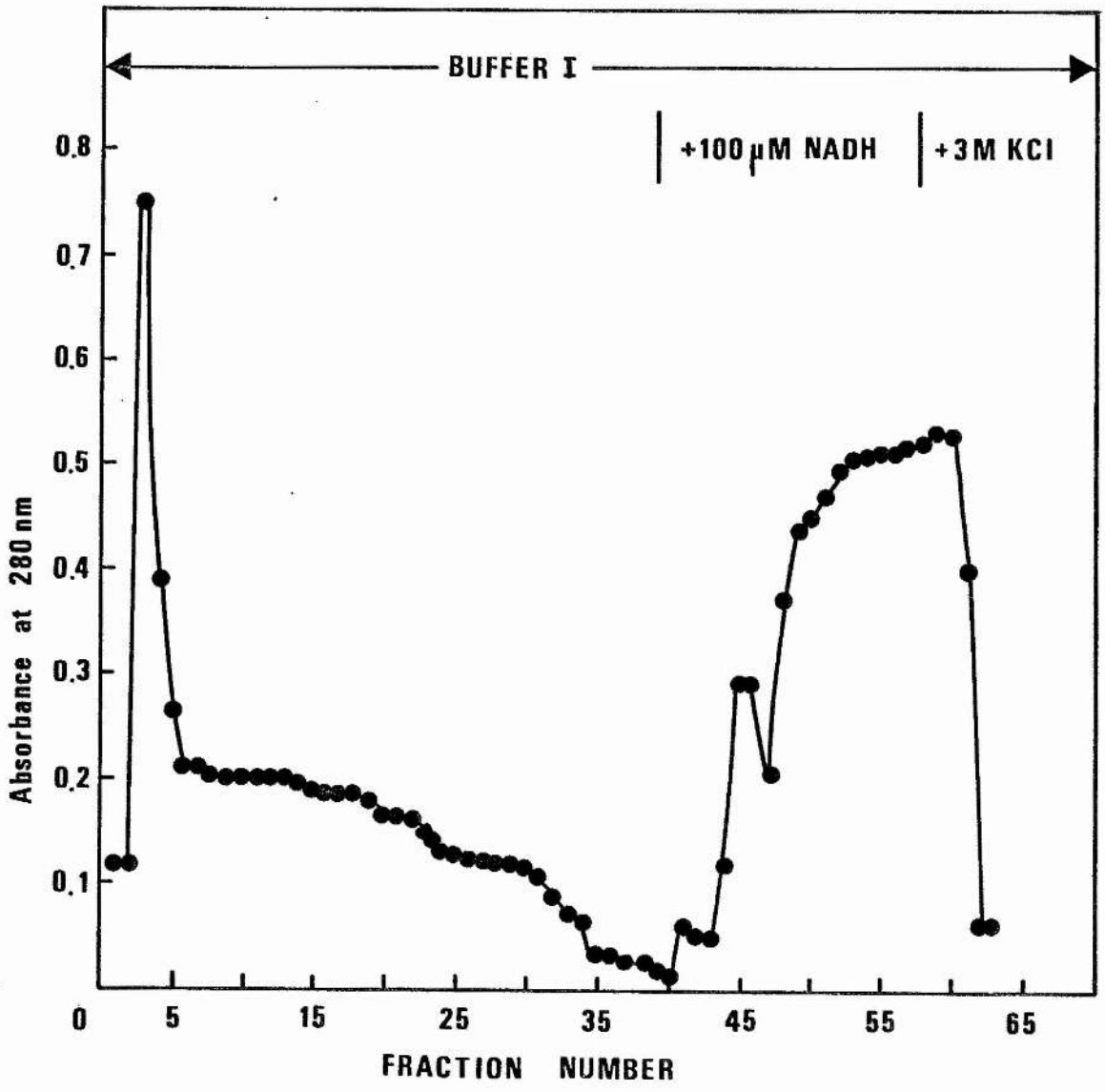
(b) Inclusion of Low Levels of KNO_3

In another attempt to increase the yield of nitrate reductase during Blue-Sepharose chromatography, the ability

FIG. 9

Elution of BSA from Blue-Sepharose with 100 μM NADH

This figure demonstrates the ability of BSA to bind to Blue-Sepharose and the ability of 100 μM NADH to elute bound BSA. 40 mg of BSA were dissolved in 8 ml of Buffer I and applied to a column (6 cm × 0.9 cm) of Blue-Sepharose. Non-absorbed protein was removed with Buffer I prior to elution with 100 μM NADH. 5 ml fractions were collected throughout.


BUFFER I
+100 µM NADH
+3M KCl
Absorbance at 280 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
15
25
35
45
55
65
FRACTION NUMBER

of low concentrations of potassium nitrate to stabilise nitrate reductase was tested. In a preliminary experiment, similar to that described for NADH in Fig. 6 , the ability of a range of concentrations of nitrate to elute nitrate reductase from Blue-Sepharose was tested (Fig. 10) It is evident (Fig. 10) that concentrations of at least 0.1 M KNO_3 are required to elute significant amounts of nitrate reductase activity. It is therefore possible to include low concentrations of KNO_3 in Buffer I to stabilise nitrate reductase without this resulting in elution of nitrate reductase from the column. The KNO_3 would have to be washed from the column immediately prior to elution with NADH in order to prevent nitrite being formed during elution as this would interfere with the assay procedure for nitrate reductase.

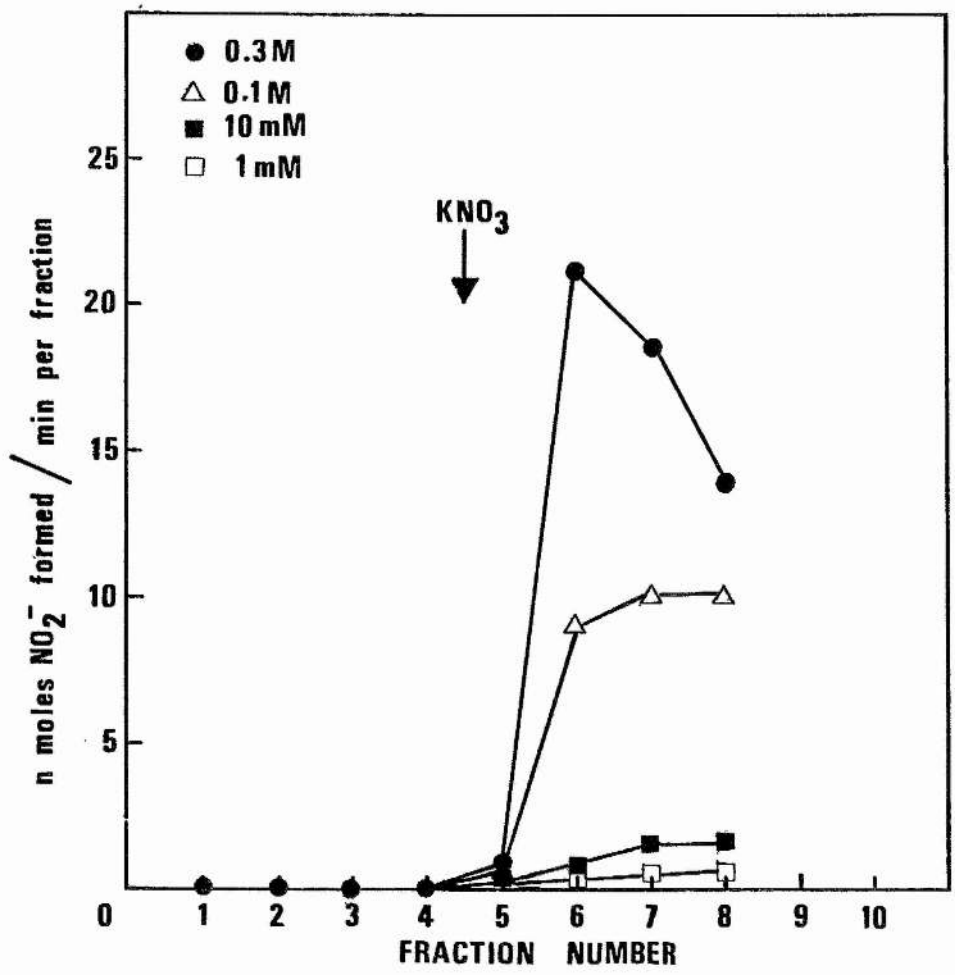
The inclusion of 10 mM KNO_3 in Buffer I prior to elution of nitrate reductase with 100 µM NADH resulted in a yield of 21%, which compares with 20% achieved with BSA and 14% without any stabilisation.

In an attempt to further raise the yield of nitrate reductase during Blue-Sepharase chromatography 40 mM KNO_3 was included in Buffer I during the removal of non-absorbed protein. This resulted in a very low continuous leakage of nitrate reductase from the column but, following removal of the KNO_3, elution with 100 µM NADH resulted in a very large peak of nitrate reductase activity (Fig. 11) which represented 37% of the applied activity. This was by far

FIG. 10

Elution of Nitrate Reductase from Blue-Sepharose with KNO_3

This figure demonstrates the ability of high concentrations of KNO_3 to elute nitrate reductase from Blue-Sepharose. Identical columns (0.8 cm × 5 cm) of Blue-Sepharose were eluted with the given concentrations of KNO_3 and 4 ml fractions were collected from each.


● 0.3 M
△ 0.1 M
■ 10 mM
□ 1 mM
KNO3
n moles NO2- formed / min per fraction
0
5
10
15
20
25
1
2
3
4
5
6
7
8
9
10
FRACTION NUMBER

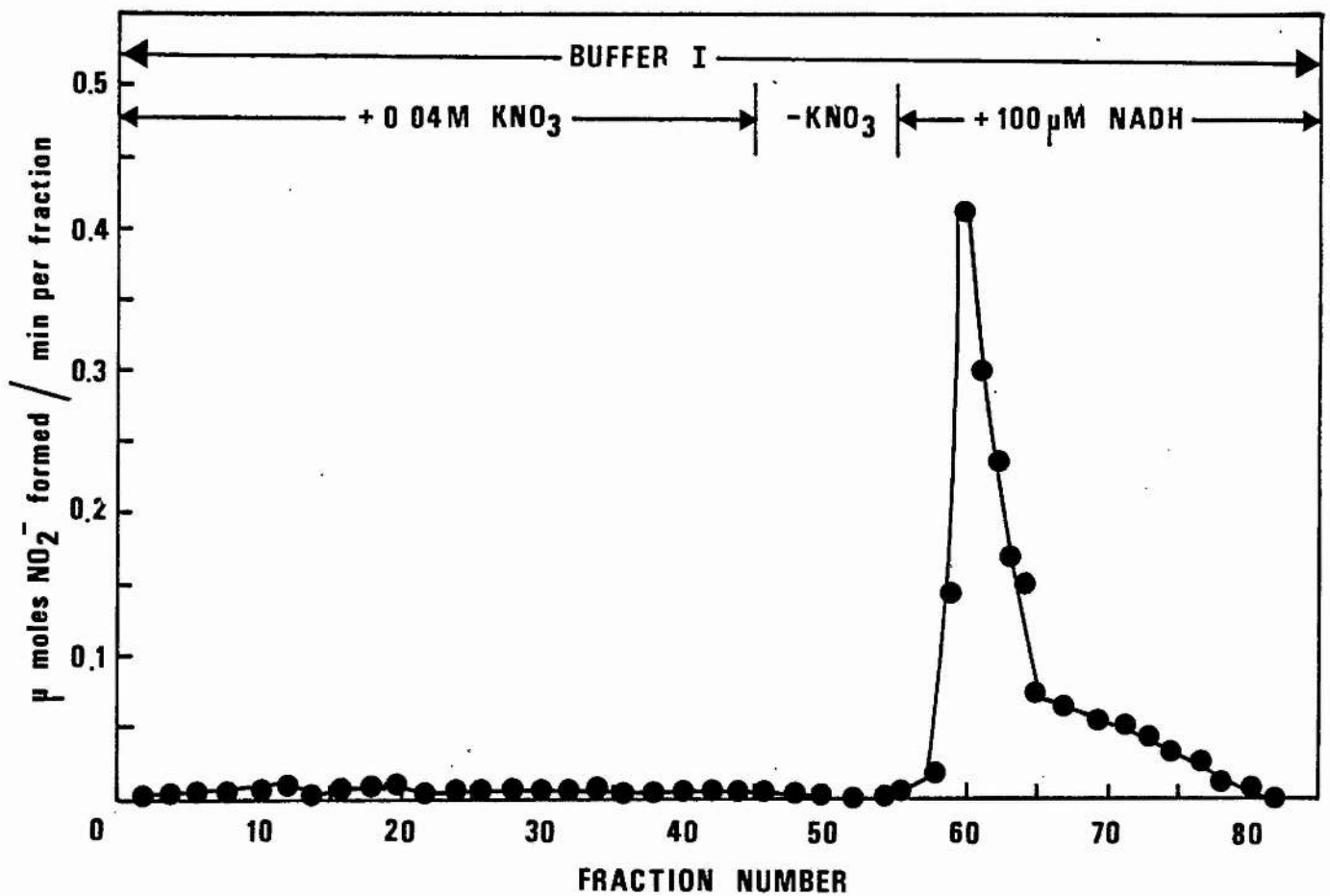

BUFFER I
+ 0 04M KNO3
-KNO3
+ 100 µM NADH
µ moles NO2- formed / min per fraction
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

FIG. 11

Stabilisation of Nitrate Reductase during Blue-Sepharose Chromatography by the Inclusion of 40 mM KNO_3

This figure shows the purification of barley nitrate reductase by Blue-Sepharose chromatography. 40 mM KNO_3 was included in buffers in order to stabilise nitrate reductase and thereby increase yield. 10 ml fractions were collected from the column (2 cm × 18 cm).

the highest yield obtained from Blue-Sepharose and details of the purification are summarised in Table 3 where it can be seen that a very high degree of purification was achieved, the peak fraction being over 1100-fold purified with a final specific activity of 2.2 units/mg.

This value is 10-fold lower than that obtained by Notton, Fido and Hewitt (1977) for spinach nitrate reductase using Blue Dextran-Sepharose. However, these authors performed several preliminary purification steps such that nitrate reductase was 120-fold purified prior to application to Blue Dextran-Sepharose. This compares with the simple 60% $(NH_4)_2SO_4$ fractionation used here which serves mainly to concentrate the sample rather than to purify nitrate reductase. It would seem likely, therefore, that significantly higher purities of barley nitrate reductase could be obtained by the inclusion of some preliminary purification procedures prior to Blue-Sepharose chromatography.

The Ability of Other Nucleotides to Elute Nitrate Reductase from Blue-Sepharose

As it had been reported (Eaglesham and Hewitt, 1971, 1975) that ADP could inhibit spinach nitrate reductase, presumably through interactions at the NADH binding site, the ability of both 1 mM and 10 mM ADP to elute nitrate reductase from Blue-Sepharose was tested. Parallel columns (0.5 cm × 8 cm) of Blue-Sepharose, equilibrated in Buffer I,

TABLE 3

PURIFICATION OF NITRATE REDUCTASE BY BLUE-SEPHAROSE CHROMATOGRAPHY

Sample	Nitrate Reductase Activity (units)*	Protein (mg)	Specific Activity (units/mg)	Fold Purification	Yield
Applied sample	4.816	2520	0.0019	1	100
Fraction 60	0.4125	0.187	2.2058	1154.8	8.56
Fraction 61	0.295	0.281	1.0498	549.6	6.125
Fraction 62	0.235	0.187	1.2566	657.9	4.879
Pooled nitrate reductase peak (Fractions 59-65)	1.797	-	-	-	-

*1 unit is defined as 1 μmole nitrite formed per minute at 25°C

were used and 2 ml of an extract prepared as described in Methods, Section II was applied to each. After removal of non-absorbed protein with Buffer I, the columns were eluted with their respective nucleotides (Fig. 12). Although no nitrate reductase activity was eluted with 1 mM ADP, high levels of activity were eluted with 10 mM ADP. It would seem probable, however, that at this concentration (used by Eaglesham and Hewitt, 1975) the ADP is acting as a structural analogue of NADH. When parallel columns were eluted with 10 mM ADP and 100 µM NADH almost identical profiles of nitrate reductase activity were obtained (Fig. 13). No elution of nitrate reductase activity was obtained with 1 mM ATP (Fig. 12) and the ability of 10 mM ATP to elute nitrate reductase was not tested.

Non-Specific Elution of Nitrate Reductase from Blue-Sepharose

(a) With KCl

Notton, Fido and Hewitt (1977) showed that although NADH was unable to elute spinach nitrate reductase from Blue Dextran-Sepharose following treatment with 0.4 M phosphate buffer, elution of nitrate reductase could be achieved with a linear gradient of 0-3M KCl. It was therefore decided to attempt to repeat this observation with barley nitrate reductase, but it was found that only 3% of the applied nitrate reductase activity was eluted by the KCl gradient, together with a large peak of protein (detected by its absorbance at 280 nm). Thus this method of elution

FIG. 12

Ability of Adenine Nucleotides to Elute Nitrate Reductase from Blue-Sepharose

This figure demonstrates that 10 mM ADP is capable of eluting nitrate reductase from Blue-Sepharose whereas 1 mM ADP and 1 mM ATP have no effect. Identical columns (0.8 cm × 5 cm) of Blue-Sepharose were used and 4 ml fractions were collected.

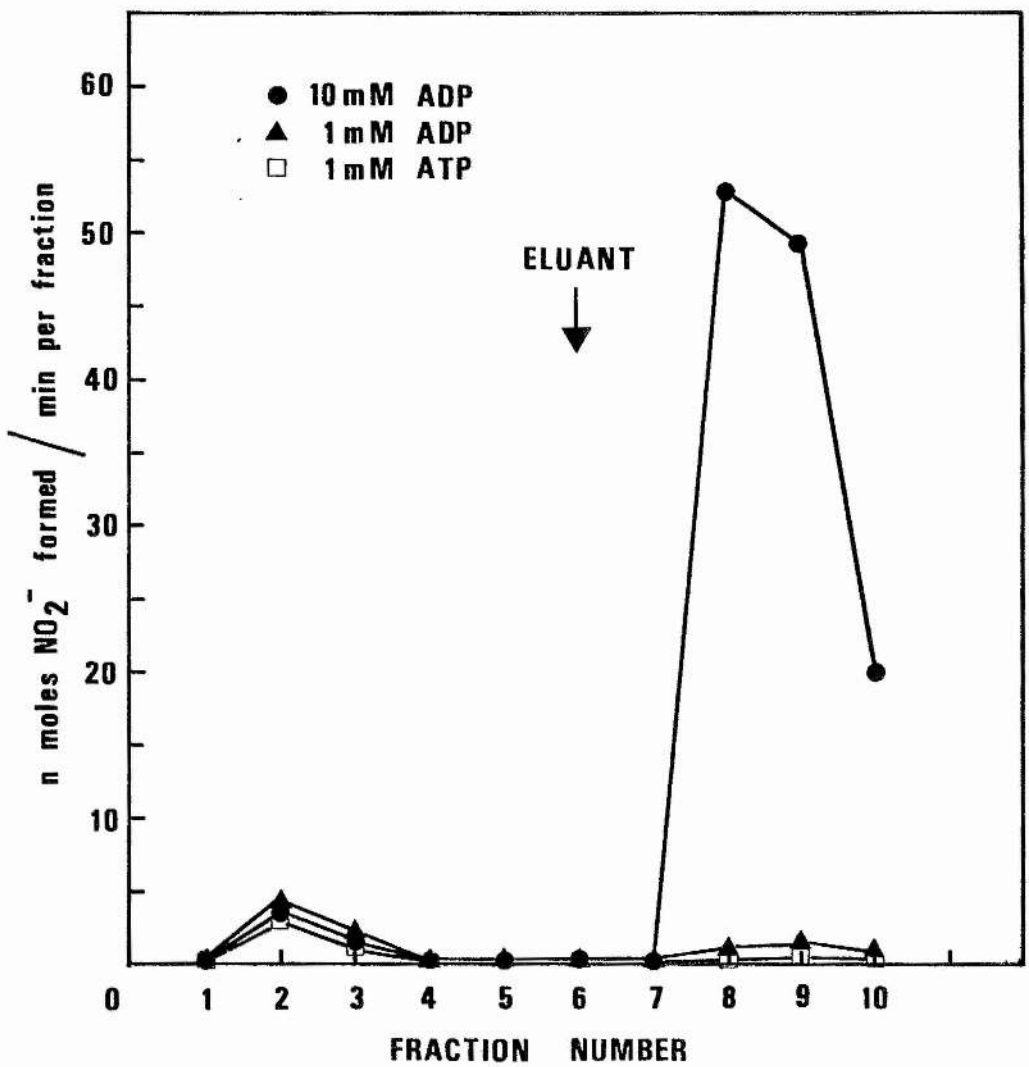
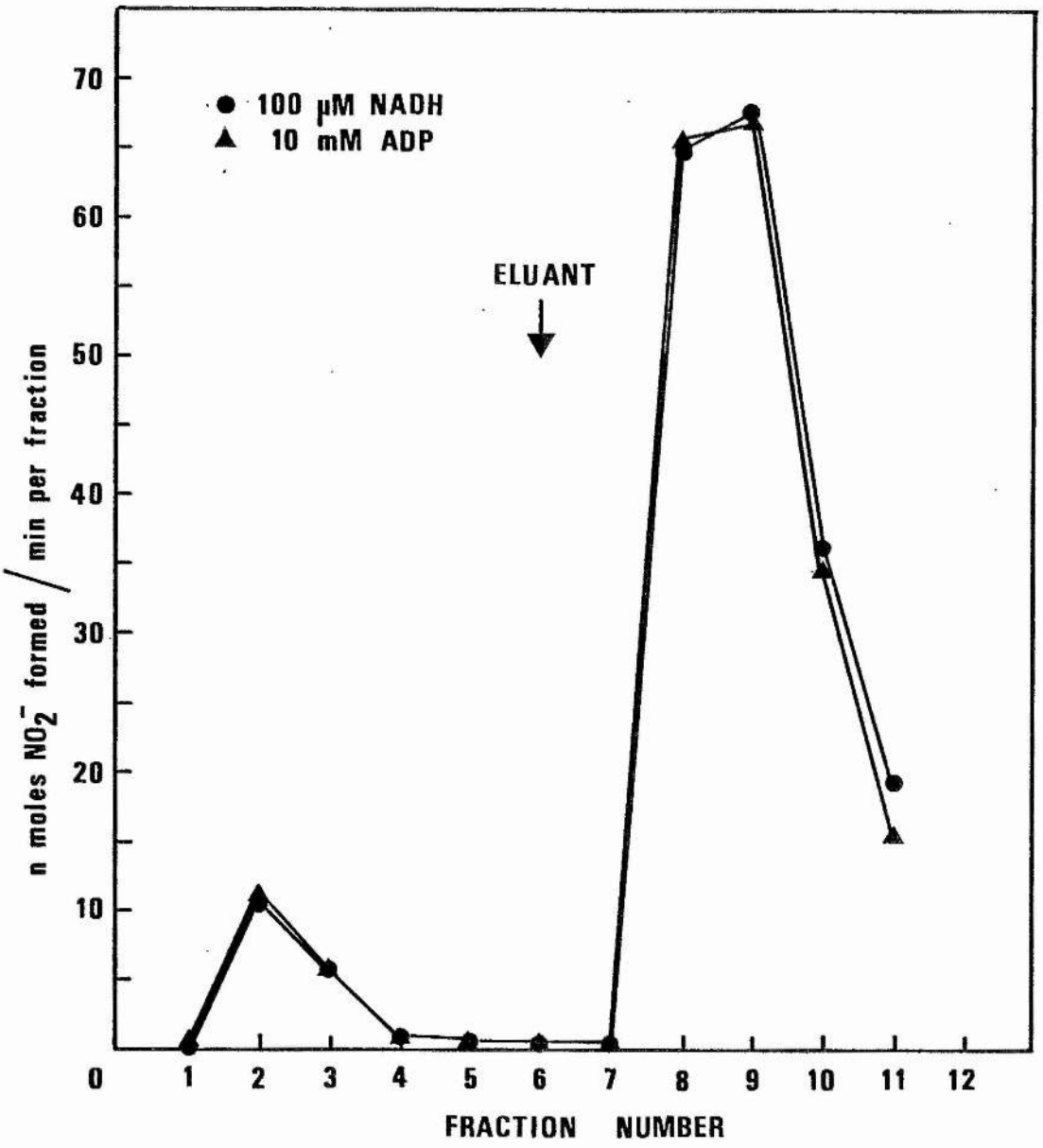

● 10 mM ADP
▲ 1 mM ADP
□ 1 mM ATP
ELUANT
n moles NO_2^- formed / min per fraction
FRACTION NUMBER
0
10
20
30
40
50
60
1
2
3
4
5
6
7
8
9
10

FIG. 13

Comparison of 100 μM NADH and 10 mM ADP as Eluants of Nitrate Reductase from Blue-Sepharose

This figure demonstrates that 100 μM NADH and 10 mM ADP (in Buffer I) are equally effective in eluting nitrate reductase from Blue-Sepharose. Identical columns (0.8 cm × 5 cm) were used and 4 ml fractions collected.


● 100 µM NADH
▲ 10 mM ADP
ELUANT
n moles NO_2^- formed / min per fraction
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
8
9
10
11
12
FRACTION NUMBER

is not specific for nitrate reductase and necessitates substantial preliminary purification of the sample to be applied to the column, as was performed by Notton, Fido and Hewitt (1977).

The very low yield of barley nitrate reductase from Blue-Sepharose following elution by KCl indicated that the KCl may have been inhibiting the enzyme. This possibility was examined (Fig. 14) using KCl concentrations equivalent to those used for elution from Blue-Sepharose. At 1M KCl, the concentration at which spinach nitrate reductase is eluted (Notton, Fido and Hewitt, 1977), the level of inhibition is at least 50%, indicating that at least 50% of the purified spinach nitrate reductase obtained by these authors is in an inactive form.

(b) With KNO_3

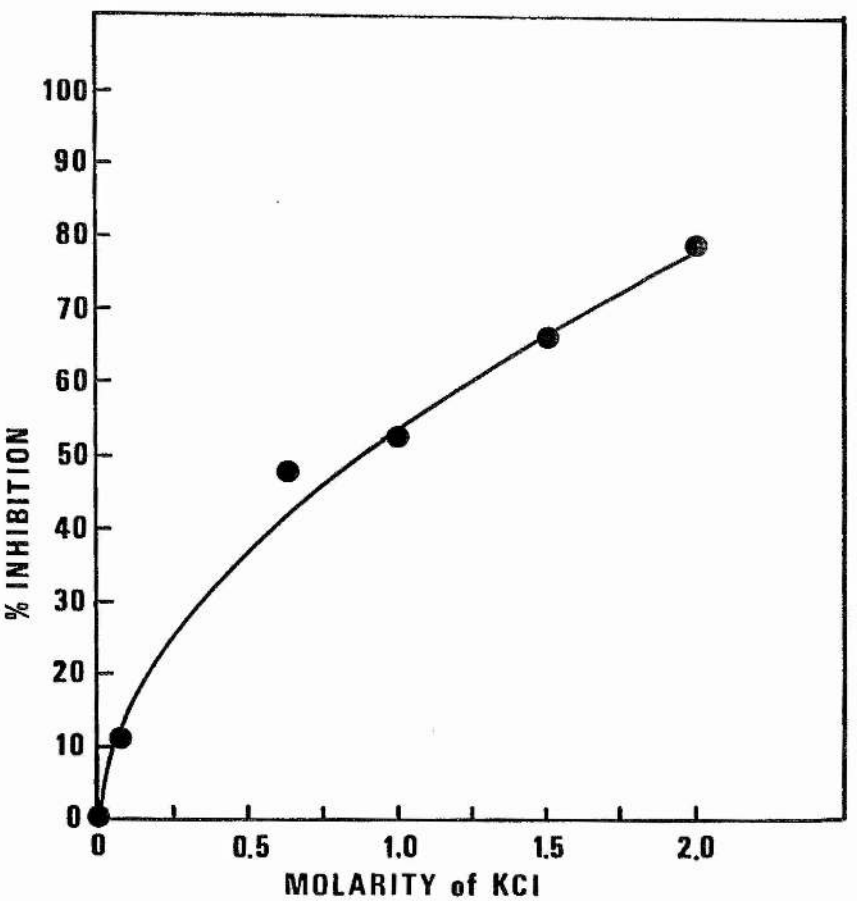
Previous results (Fig. 10) had indicated that 0.3 M KNO_3 was capable of eluting nitrate reductase from Blue-Sepharose and so it was decided to compare the efficiency of this method with that using KCl as eluant, as it is likely that both methods function by raising the ionic strength.

50 g of barley shoots were harvested and an extract prepared as described in Methods, Section II. Nitrate reductase was precipitated from the supernatant by adjustment to 60% saturation with $(NH_4)_2SO_4$, dissolved in 6 ml of Buffer I and applied to a column (1.5 cm × 10 cm) of

FIG. 14

Inhibition of Nitrate Reductase by KCl

This figure shows the inhibition of barley nitrate reductase caused by addition of various concentrations of KCl. All nitrate reductase assays were performed 2 minutes after addition of KCl and the final concentrations of KCl are given.


100
90
80
70
60
50
40
30
20
10
0
% INHIBITION
0
0.5
1.0
1.5
2.0
MOLARITY of KCl

Blue-Sepharose equilibrated in Buffer I. Non-absorbed protein was removed from the column with 140 ml of Buffer I, following which the column was eluted with 100 ml 0.3 M KNO_3 in Buffer I and then 70 ml of 3M KCl in Buffer I to elute any remaining protein. The recovery of nitrate reductase activity (Fig. 15) was 75% which compares with only 3% found previously for elution with KCl. Thus it is clear that if sufficient preliminary purification of nitrate reductase is undertaken so that this is virtually the only protein present in the sample capable of binding to Blue-Sepharose, high yields of active nitrate reductase could be obtained from Blue-Sepharose by elution with KNO_3 but not with KCl (as used by Notton, Fido and Hewitt, 1977).

SECTION III - PRELIMINARY PURIFICATION PROCEDURES

The need for preliminary purification procedures has already been mentioned. Without these, the amount of protein to be applied to Blue-Sepharose would necessitate the biggest of columns to be used as it is likely that at least 1 kg of tissue would be required to obtain significant amounts (2-3 mgs) of nitrate reductase (Notton, Fido and Hewitt, 1977). Also, it is unlikely that homogeneous nitrate reductase could be obtained from Blue-Sepharose by elution with 100 µM NADH without the inclusion of some preliminary purification steps. An assessment of some purification procedures is given below.

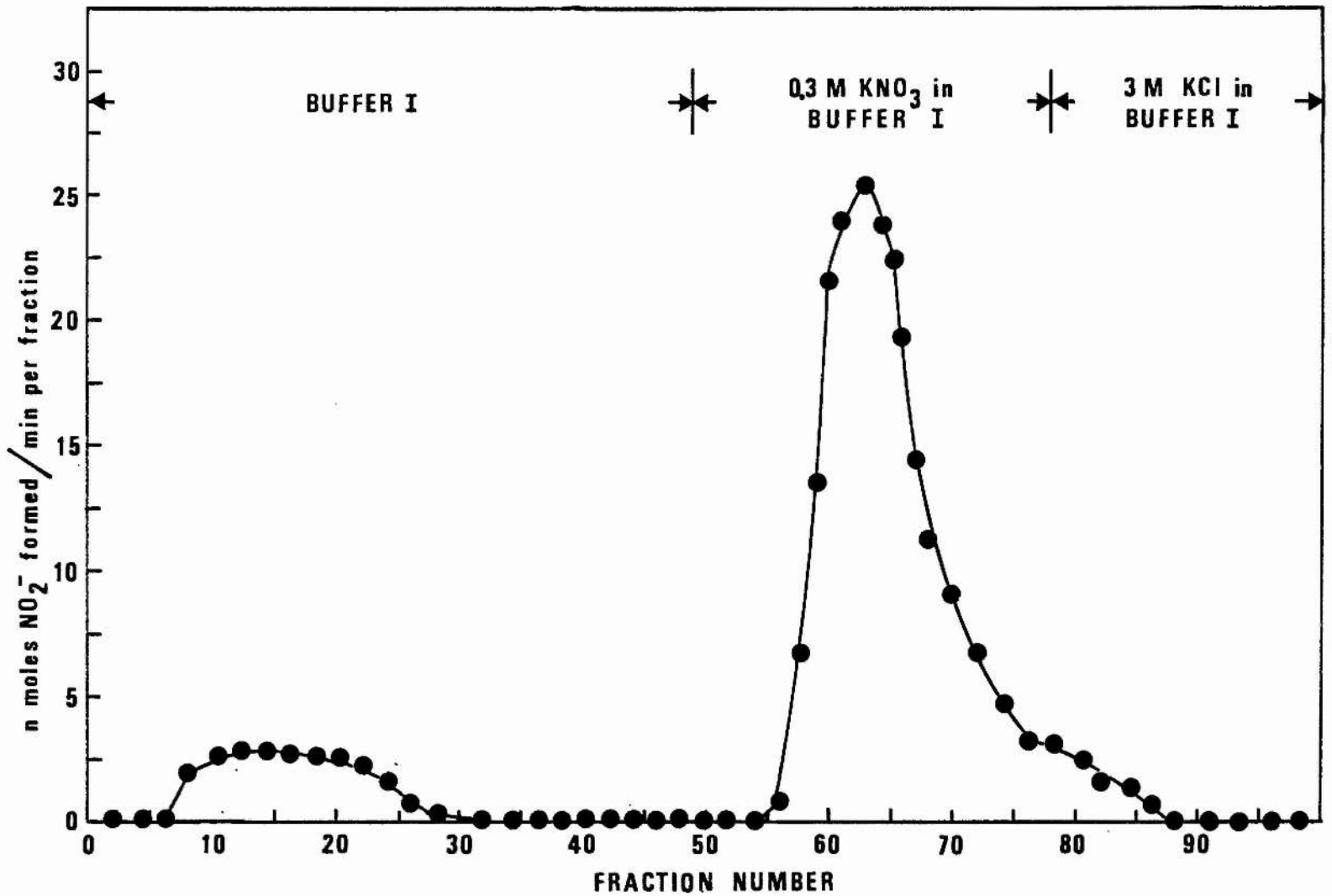

BUFFER I
0,3 M KNO3 in BUFFER I
3 M KCl in BUFFER I
n moles NO2- formed/min per fraction
FRACTION NUMBER
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70
80
90

FIG. 15

Elution of Nitrate Reductase from Blue-Sepharose with 0.3 M KNO_3

This figure demonstrates that nitrate reductase activity can be eluted from Blue-Sepharose with a high yield by elution with 0.3 M KNO_3 contained in Buffer I. No additional activity can be eluted by subsequent elution with 3 M KCl. 3.2 ml fractions were collected from the column (1.5 cm × 10 cm).

Extraction of Nitrate Reductase from Large Amounts of Barley

The experiments described so far have all utilised small amounts of barley which could be homogenised quickly and efficiently by means of a mortar and pestle. However, the purification of spinach nitrate reductase reported by Notton, Fido and Hewitt (1977) utilised 1 kg of shoots and only yielded 0.4 mg of protein, indicating that to purify mg quantities of nitrate reductase several kilos of tissue must be used.

The efficiency of three extraction procedures was therefore evaluated:

(a) Extraction with a Mortar and Pestle

This was the most laborious and time-consuming method, but consistently gave the best results. When tissue was ground in Buffer I (3 ml buffer per gram tissue) and the cell debris removed by filtration through a double layer of muslin prior to centrifugation, high levels of both nitrate reductase activity and protein were obtained.

(b) Mechanical Homogenisation with a Waring Blender

This method of extraction was found to be unsuitable for use with barley shoots. Due to the thin shape of the leaves and their small size (4 cm when nitrate reductase activity is greatest - see later) large numbers of shoots remain uncut. Also, due to the high speed of the blades,

a significant amount of frothing could not be prevented resulting in denaturation of extracted proteins. No nitrate reductase activity could be detected in extracts prepared this way.

(c) Liquid Nitrogen - Mediated Cell Breakage Followed by Vigorous Mixing with Buffer I

In this method, shoots were harvested and then frozen by addition of liquid nitrogen. The brittle shoots resulting from this treatment were then crushed to a powder in a large mortar to which was then added the required amount of Buffer I (3 ml buffer per gram tissue). This 'soup' was then vigorously mixed with a large pestle and then stirred at 4°C for 30 minutes.

A direct comparison of the efficiency of methods (a) and (c) is given below in Table 4 in which 400 g of tissue was subjected to each method of extraction, cell debris removed by filtration through a double layer of muslin and the nitrate reductase and protein levels measured in the resulting filtrates.

TABLE 4

Method of Extraction	Total Nitrate Reductase Activity (units)*	Total Protein (mg)
(a)	23.42	7134
(c)	12.881	4613

*1 unit is defined as 1 μmole nitrite formed per minute at 25°C

It is evident from these results that method (a) (mortar and pestle) is much more efficient in the extraction of nitrate reductase activity and protein than is method (c) (liquid nitrogen-assisted). A further problem encountered with method (c) was that subsequent centrifugation resulted in precipitation of almost 50% of the nitrate reductase activity. In all subsequent work, therefore, barley shoots were extracted by method (a) using a mortar and pestle.

Streptomycin Sulphate Treatment

As indicated earlier (Section II), treatment of a barley extract with protamine sulphate, at the concentration suggested by Solomonson (1975), resulted in significant losses of nitrate reductase activity. An alternative method of removing nucleic acid material from the extract was suggested by Notton, Fido and Hewitt (1977) and involved the use of streptomycin sulphate. Streptomycin sulphate was added to the filtered extract (5 mg streptomycin sulphate per gram of tissue extracted) prior to centrifugation and the pH restored to 7.5 if necessary by the addition of dilute NaOH. After stirring at 4°C for 10 minutes, nucleic acid material and cell debris were removed together by centrifugation at 38-40 000 g for 50 minutes, the resultant supernatant containing the nitrate reductase activity.

During this centrifugation, approximately 30% of the protein precipitated. Nitrate reductase activity was some-

times found to increase at this stage by as much as 10% but an several occasions was found to decrease, exceptionally by as much as 20%. The reasons for the variability found at this, and other stages of the purification, will be examined in the Discussion at the end of this Chapter.

Ammonium Sulphate Fractionation

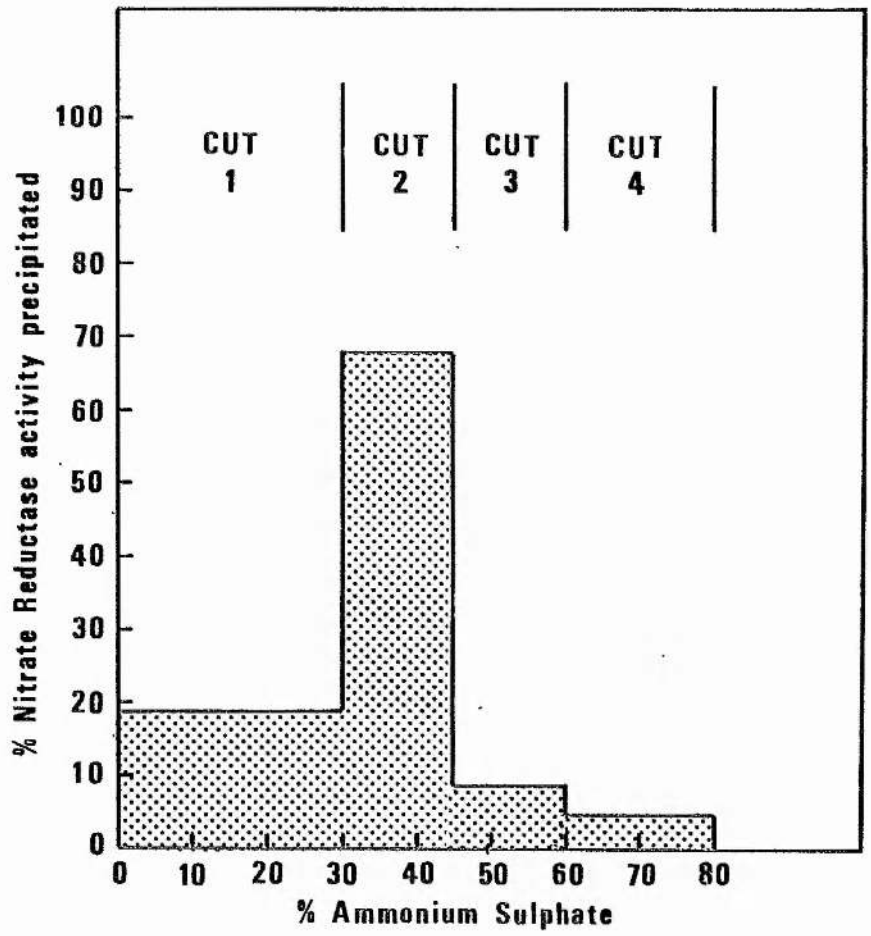
In early experiments (Section II), $(NH_4)_2SO_4$ treatment was used solely as a means of concentrating the protein samples for subsequent chromatography. It is likely, however, that a careful choice of $(NH_4)_2SO_4$ concentrations would itself provide some purification of the enzyme in addition to functioning as a concentration stage. A 50 g sample of barley shoots was therefore harvested, nitrate reductase extracted by grinding the shoots with Buffer I in a mortar, and cell debris removed by centrifugation. A series of $(NH_4)_2SO_4$ fractionations was then carried out on the extract at $(NH_4)_2SO_4$ concentrations of 30%, 45%, 60% and 80%. At each stage, the solution was stirred for 30 minutes at 4°C and precipitated protein then collected by centrifugation at 20 000 g for 20 minutes, dissolved in 10 ml of Buffer I and then assayed for nitrate reductase activity (Fig. 16).

The bulk (67.8%) of the nitrate reductase activity was found to precipitate between 30% and 45% saturation with $(NH_4)_2SO_4$ (Fig.16) while a further 18.9% was precipitated by 30% $(NH_4)_2SO_4$. However, only 10% of the protein

FIG. 16

$(NH_4)_2SO_4$ Precipitation Characteristics of Barley Nitrate Reductase

This figure illustrates the required $(NH_4)_2SO_4$ concentrations for precipitation of barley nitrate reductase. Experimental details are given in the main text.


CUT 1
CUT 2
CUT 3
CUT 4
% Nitrate Reductase activity precipitated
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
% Ammonium Sulphate

is precipitated by 30% $(NH_4)_2SO_4$, including most of the green-coloured, high molecular weight, material in the extract. Thus, although it would be useful to have the green-coloured contaminants removed from the sample the corresponding loss of 20% of the nitrate reductase activity is undesirable. Indeed, when this step was attempted with an extract prepared from 500 g of barley shoots, it was found that 35% of the nitrate reductase was precipitated by 30% $(NH_4)_2SO_4$.

Routinely, therefore, nitrate reductase activity was concentrated by collecting a 0-45% $(NH_4)_2SO_4$ precipitate which was then used for further purification. This precipitate usually contained 5-7 mg of protein per gram of tissue extracted and 60-90% of the initial nitrate reductase activity. The sample was, however, very green necessitating the removal of these coloured components at a subsequent stage of the purification.

Gel Filtration through Biogel A1.5 m

Preliminary experiments indicated that the green-coloured components in an extract from barley shoots were eluted at the void volume following gel filtration of an extract through Biogel A1.5 m. It was therefore decided to use this step to separate these components from nitrate reductase which is significantly retarted during filtration Biogel A1.5 m.

The largest column available for use was 4.1 cm × 108 cm

and contained almost 1500 ml of swollen gel. However, the maximum size of sample which this column could process to obtain separation of nitrate reductase from the green-coloured components was 20 ml. This was the volume of sample derived from 250 g of tissue following a 0-45% $(NH_4)_2SO_4$ fractionation, and routinely contained 1500 mg of protein. It was therefore not possible to process more than 250 g of barley shoots in any one experiment.

The distributions of nitrate reductase activity and protein from such a column are presented in Fig. 17. Virtually all the high molecular weight green components were eluted by fraction 12 (Fig. 12) while the major nitrate reductase peak encompassed fractions 14-26 which were pooled for further purification. A small peak of nitrate reductase activity is frequently observed associated with the high molecular weight components eluting just after the void volume (Fig. 17). However, this peak is not observed during small-scale gel filtration procedures and it is therefore likely to be due to non-specific protein associations caused by high protein concentration rather than to the existence of a separate high molecular weight form of nitrate reductase.

It was usually possible to recover 70% of the applied nitrate reductase activity associated with less than 20% of the applied protein from this gel filtration step, yielding a purification of 4-5 fold. The major importance of this step is, however, the removal of the high molecular

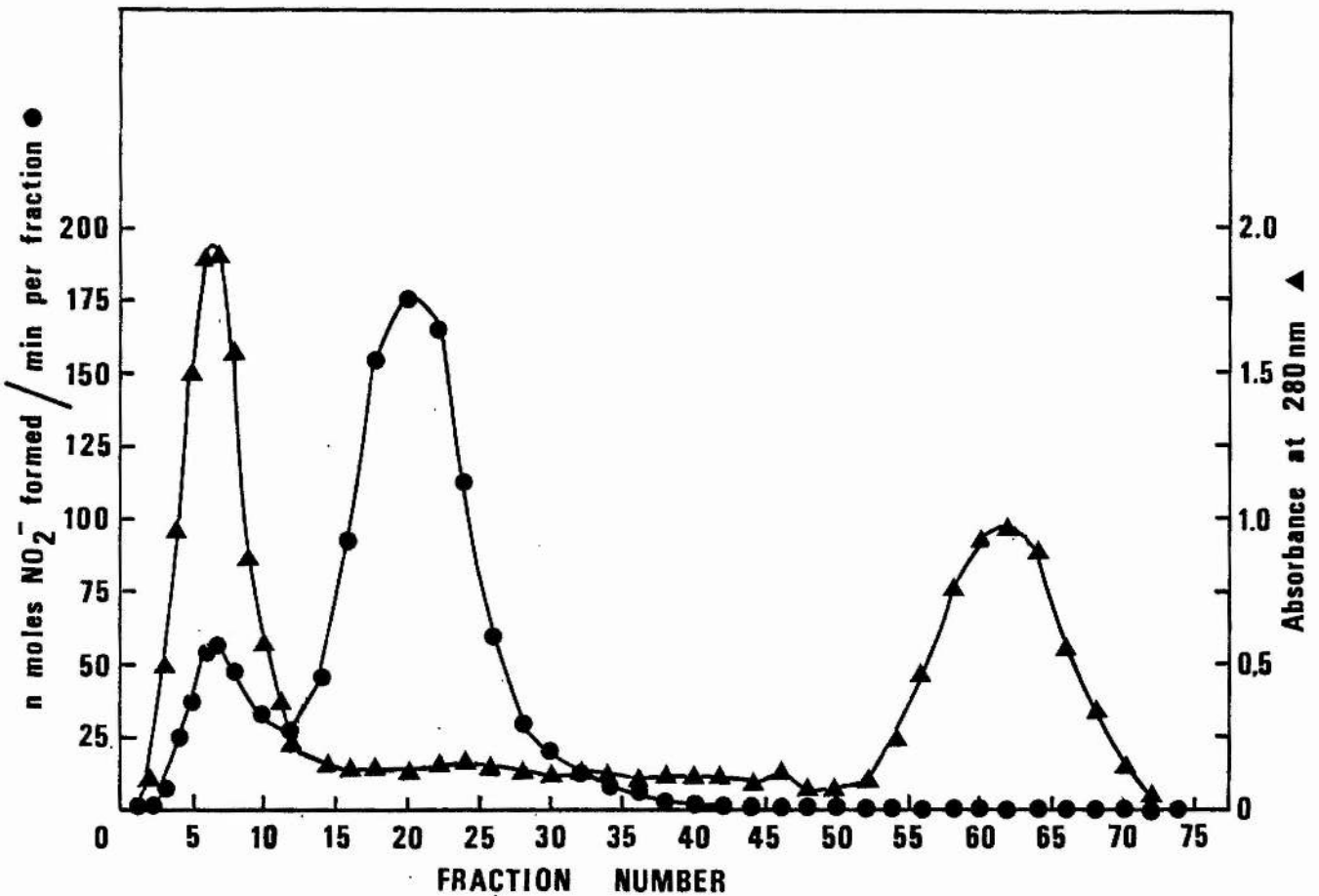

n moles NO_2^- formed / min per fraction ●
Absorbance at 280 nm ▲
FRACTION NUMBER
0
25
50
75
100
125
150
175
200
0
0.5
1.0
1.5
2.0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75

FIG. 17

Gel Filtration of a Barley Extract Through Biogel A1.5 m

This figure shows the separation of nitrate reductase activity from the major protein peaks by gel filtration through Biogel A1.5 m as described in Methods, Section II. Samples were diluted 5-fold prior to determination of the absorbance at 280 nm.

weight green components from the sample, as these would interfere with subsequent purification techniques. Prior to storage (see below) the pooled fractions from Biogel A1.5 gel filtration were concentrated by $(NH_4)_2SO_4$ fractionation and dissolved in a small volume of Buffer I.

A summary of the cumulative purification achieved by the steps so far described is presented in Table 5.

SECTION IV - STORAGE OF NITRATE REDUCTASE SAMPLES

As was indicated at the start of Section III, several kilos of barley would have to be processed in order to obtain a few mgs of nitrate reductase but, as shown in the previous section, only 250 g of tissue could be processed in any one experiment. It was therefore necessary to find a method of storing nitrate reductase samples so that the enzyme from separate 250 g batches could be pooled prior to further purification.

Solomonson *et al.* (1975) have reported that *Chlorella* nitrate reductase can be stored indefinitely at -15°C in a buffer containing 40% glycerol to prevent freezing. Storage of barley nitrate reductase samples under the same conditions resulted in significant losses of activity, but duplicate samples stored at -70°C were found to be much more stable (Fig. 18) despite the complete freezing of the solution. After ten days of storage, twice as much activity was present in the -70°C sample compared with the -15°C sample. After an initial decline in activity (Fig. 18),

TABLE 5

PRELIMINARY PURIFICATION OF BARLEY NITRATE REDUCTASE

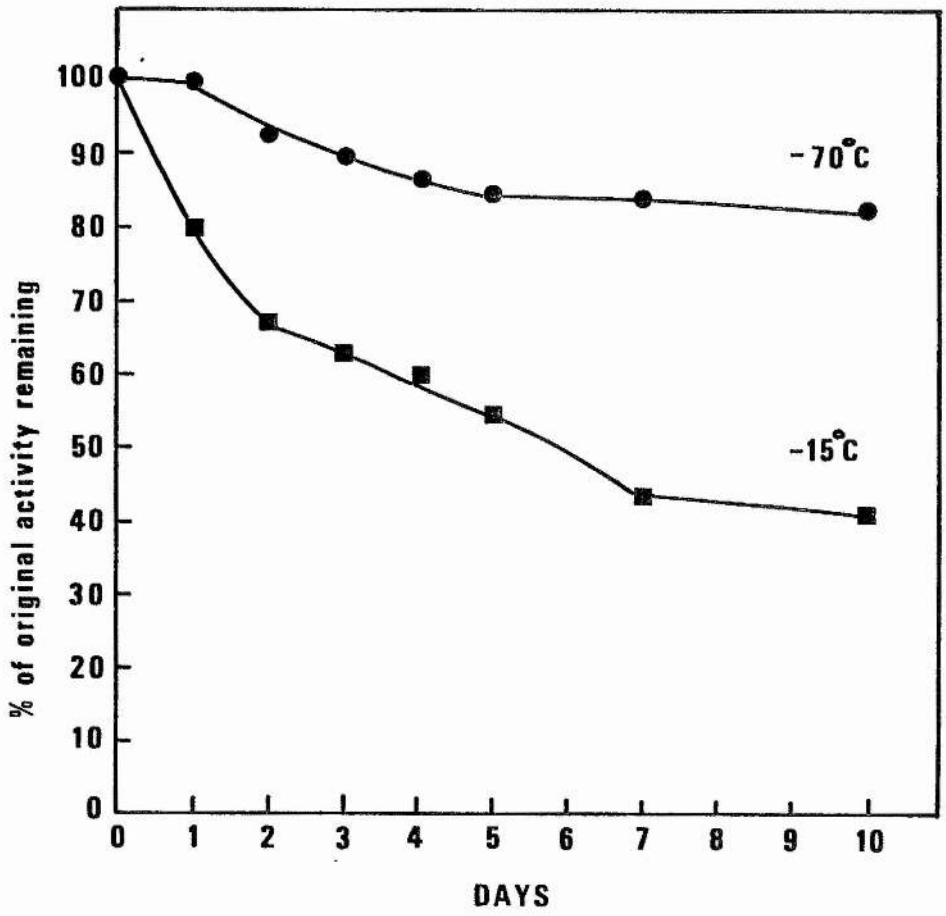
	Nitrate Reductase Activity (units)*	Protein (mg)	Specific Activity (units/mg)	Fold Purification	Yield
Filtered Extract	15.238	3665	0.004	1	100
Streptomycin Sulphate	16.537	2554	0.0065	1.6	108
0-45% Ammonium Sulphate	14.625	1546	0.0095	2.4	95.9
Biogel A1.5 m Filtration	10.175	245	0.041	10.25	66.8
0-50% Ammonium Sulphate	8.84	178	0.051	12.75	58

* 1 unit is defined as 1 µmole nitrate reduced per minute at 25°C.

FIG. 18

Storage of Nitrate Reductase

This figure shows the recovery of nitrate reductase activity following storage of samples for the times indicated at either -70°C or -15°C in Buffer I (pH 7.5 at 20°C) adjusted to contain 40% (v/v) glycerol.


% of original activity remaining
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
DAYS
-70°C
-15°C

the -70°C sample becomes almost completely stable and many samples stored for a period of two months at -70°C in buffer containing 40% glycerol were found to retain 85-90% of their original nitrate reductase activity.

Storage of nitrate reductase samples at -70°C in the absence of glycerol resulted in an almost complete loss of enzyme activity over a 24-hour period whilst the presence of 10% glycerol reduced these losses to about 50% over a 24-hour period.

Consequently, when storage of a sample was necessary, the sample was adjusted to 40% saturation with glycerol and then placed at -70°C. Prior to further use the sample was thawed and glycerol removed by precipitation of the protein with $(NH_4)_2SO_4$ (see Methods, Section II).

SECTION V - DEAE-CELLULOSE CHROMATOGRAPHY

The major purification step used for higher plant nitrate reductase before the advent of Blue-(Dextran)-Sepharose chromatography was adsorption chromatography using calcium phosphate gel or hydroxylapatite (Notton and Hewitt, 1971a). However, this material is both expensive and, due to its extreme fragility can only be used once, and so an alternative, more economical, procedure was examined namely DEAE-Cellulose ion-exchange chromatography.

The elution characteristics of nitrate reductase from DEAE-cellulose were examined by extracting the protein from

6 g of barley shoots (see Methods, Section II), removing cell debris by centrifugation and applying the supernatant directly to a column (1 cm × 6.3 cm) of DEAE-cellulose pre-equilibrated in Buffer I lacking EDTA (to prevent interference in the ion-exchange process). The column was then washed with two column-volumes (40 ml) of each of a series of buffers of increasing ionic strength, as indicated below in Table 6. All buffers were potassium phosphate, pH 7.5, containing 10 μM FAD and 1 mM cysteine.

TABLE 6

Treatment	Nitrate Reductase Activity Eluted (units × 10^3)
40 ml 0.05 M Buffer (Buffer I lacking EDTA)	0
40 ml 0.10 M Buffer	0.75
40 ml 0.12 M Buffer	14
40 ml 0.14 M Buffer	11.8
40 ml 0.16 M Buffer	3.83
40 ml 0.18 M Buffer	3.92

* 1 unit is defined as 1 μmole nitrite formed per minute at 25°C.

Thus, barley nitrate reductase can be bound to and eluted from DEAE-cellulose which contrasts with the results obtained with spinach nitrate reductase (B.A. Notton,

personal communication) which binds irreversibly with DEAE-cellulose.

To determine the extent of purification achieved by this step, 30 g of barley shoots were ground in a mortar with 90 ml of Buffer I and, after centrifugation to remove cell debris, the supernatant was adjusted to 80% saturation with $(NH_4)_2SO_4$. (This precipitates nearly all the protein and was done for two reasons: (a) to concentrate the sample, and (b) to ensure that all NADH-cytochrome c reductase species were present in the sample (see Chapter 2 of these Results) so that the ability of DEAE-cellulose to separate these species could be tested.) The resulting precipitate was collected by centrifugation, resuspended in 20 mM potassium phosphate buffer pH 7.5 (to ensure binding of all NADH-cytochrome c reductase species) containing 10 µM FAD and desalted by passage through a column (4.1 cm × 50 cm) of Sephadex G25 preequilibrated with the same buffer. Fractions containing protein were pooled (30 ml) and applied to a column (1.5 cm × 9 cm) of DEAE-cellulose which was then washed with a further 70 ml of buffer. Enzyme activity was eluted with a 400 ml linear gradient of from 20 mM to 300 mM potassium phosphate buffer, pH 7.5, containing 10 µM FAD and 5 ml fractions were collected throughout (Fig. 19). Nitrate reductase activity co-eluted with the major NADH-cytochrome c reductase species, as expected, and with a yield of 80% of the applied activity. Fractions which contained the highest levels of nitrate reductase activity (Fig. 19) were pooled and the protein content

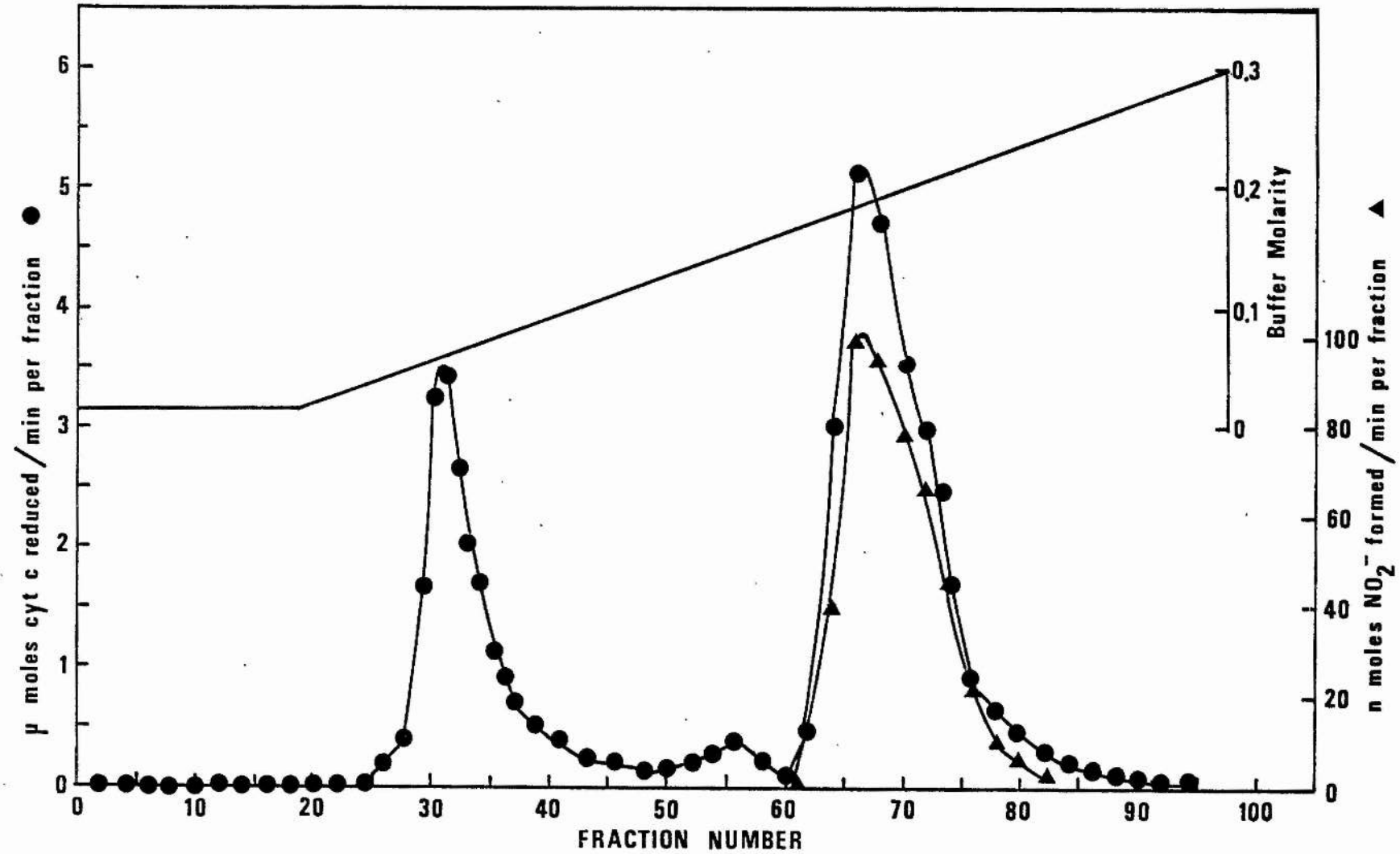

μ moles cyt c reduced / min per fraction ●
n moles NO_2^- formed / min per fraction ▲
Buffer Molarity
FRACTION NUMBER
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
0
0,1
0,2
0,3
0
20
40
60
80
100

FIG. 19

Elution of Nitrate Reductase from DEAE-Cellulose

This figure demonstrates that barley nitrate reductase can be eluted from DEAE-cellulose by an increase in the buffer concentration. This method is also effective in separating nitrate reductase from other NADH-cytochrome c reductase species in the extract. 5 ml fractions were collected from the column (1.5 cm × 9 cm).

determined (Table 7).

Thus DEAE-cellulose chromatography is a relatively good method of purifying barley nitrate reductase, the final specific activity reported here (Table 7) being comparable with that obtained following Biogel filtration (Table 5). Problems were encountered, however, when scaling the experiment up to deal with larger amounts of protein, the major one of which was the time required to elute non-absorbed protein from the column. With a sample equivalent to that applied to Biogel A1.5 m (i.e. derived from 250 g of shoots and subjected to streptomycin sulphate treatment and $(NH_4)_2SO_4$ fractionation at 45% saturation) it took 12 hours to reduce the E_{280} of the eluate to less than 0.01 after which time only 35% of the applied nitrate reductase activity could be recovered. After several unsuccessful attempts to increase this yield it was concluded that DEAE-cellulose chromatography was best suited to the rapid purification of relatively small enzyme samples as the time required for larger scale operations resulted in significantly lowered yields of nitrate reductase.

Two minor points are also worth noting. The first of these is that the FAD used in eluant buffers binds to DEAE-cellulose such that the column becomes a deep orange colour. This does not, apparently, affect the subsequent binding of protein to the column. The second point is that the high molecular weight, green-coloured components present in the applied sample were found to bind irreversibly and could not be eluted even with 3 M KCl.

TABLE 7

PURIFICATION OF NITRATE REDUCTASE BY DEAE-CELLULOSE CHROMATOGRAPHY

	Nitrate Reductase Activity (units)*	Protein (mg)	Specific Activity (units/mg)	Fold Purification	Yield
Centrifuged Extract	1.35	450	0.003	1	100
0-80% $(NH_4)_2SO_4$	1.2	400	0.003	1	89
DEAE-Cellulose (pooled fractions 64-74)	0.787	15	0.052	17.5	60.5

* 1 unit is defined as 1 μmole nitrite produced per minute at 25°C.

SECTION VI - FURTHER PURIFICATION OF BARLEY NITRATE REDUCTASE

Because of interests in other aspects of nitrate reductase it was not until early 1979 that further concerted efforts were made to obtain highly purified barley nitrate reductase. By this time there were several reports in the literature on the purification of higher plant nitrate reductase and these are summarised in Table 8 where it can be seen that the highest specific activities recorded have all been obtained using Blue Dextran-Sepharose rather than commercially available Blue-Sepharose which I had used previously. Indeed, the only report using commercial Blue-Sepharose is that of Notton, Fido, Watson and Hewitt (1979) who only obtained a specific activity of 0.85 units/mg for spinach nitrate reductase. This compares with the 24.12 units/mg achieved by the same authors with the same enzyme using Blue Dextran-Sepharose.

As indicated in the Introduction to this Chapter, the major difference between these two affinity media is the absence of the dextran spacer molecule from commercial Blue-Sepharose. The results reported in the literature (Table 8) would suggest that this omission is highly significant as far as the purification of higher plant nitrate reductase is concerned. It was decided, therefore, to attempt to purify barley nitrate reductase using Blue Dextran-Sepharose.

TABLE 8

HIGHER PLANT NITRATE REDUCTASES PURIFIED BY AFFINITY CHROMATOGRAPHY

	Final Specific Activity (Units/mg)	Fold Purification	Affinity Medium Used	Reference
Spinach	24.12	2600	Blue-Dextran Sepharose	Notton, Fido & Hewitt (1977)
Wheat	23.1	2573	Blue-Dextran Sepharose	Sherrard & Dalling (1979)
Spinach	22.0	1600	Blue-Dextran Sepharose	Guerrero, Jetschmann & Volker (1977)
Corn	6.9	690	Blue Sepharose	Campbell & Smarrelli, Jr. (1978)
Squash (*Cucurbita pepo*)	1.9	154	Blue Sepharose	Campbell & Smarrelli, Jr. (1978)
Maize Scutellum	1.3	140	Blue-Dextran Sepharose	Campbell (1978)
Spinach	0.85	94.5	Blue Sepharose	Notton, Fido, Watson & Hewitt (1979)
Barley	0.49	109	NADH-Sepharose	Heimer, Krashin & Riklis (1976)

Binding and Elution Characteristics of Barley Nitrate Reductase with Blue Dextran-Sepharose

Blue Dextran-Sepharose was prepared by the method of Sherrard and Dalling (1979), as described in Methods, Section IV. Some problems were encountered initially as it was found that barley nitrate reductase, contained in Buffer I, did not bind to Blue Dextran-Sepharose. This was overcome by reducing the phosphate concentration of the buffer to 0.02 M (Buffer I is 0.05 M) while retaining the other components of Buffer I, namely 0.1 mM EDTA, 1 mM cysteine and 10 µM FAD. This 0.02 M buffer will be referred to as Buffer III and was used in all subsequent experiments with Blue Dextran-Sepharose.

Sherrard and Dalling (1979) reported that 5 µM NADH could elute wheat nitrate reductase from Blue Dextran-Sepharose, as had originally been shown by Solomonson (1975) for *Chlorella* nitrate reductase. The optimum NADH concentration for the elution of barley nitrate reductase from Blue Dextran-Sepharose was determined using parallel columns each containing 5 ml of gel. Identical 1 ml enzyme samples (purified by streptomycin sulphate treatment, $(NH_4)_2SO_4$ fractionation and Biogel A1.5 m gel filtration) were applied to each of the columns which were then washed with 5 column-volumes (25 ml) of Buffer III to remove non-absorbed protein. Nitrate reductase activity was then eluted from each of the columns with 20 ml of either 5 µM- 10 µM- or 50 µM-NADH in Buffer III. Each column was then

washed with 10 ml of Buffer III lacking NADH following which each was eluted with 20 ml of 0.3 M KNO_3 in Buffer III in order to elute any nitrate reductase still bound to the columns (see Section II). A fourth column was washed in an identical manner but NADH was replaced by 0.1 M KNO_3. The results of this experiment are presented in Table 9 and Fig. 20.

Elution with NADH released nitrate reductase from all three columns (numbers 1-3, Table 9), the lowest NADH concentration (5 μM) resulting in the highest level of nitrate reductase activity (35.5% of the applied activity). However, high levels of nitrate reductase were eluted from these columns by the subsequent elution with 0.3 M KNO_3. This indicates that a significant amount of the nitrate reductase bound to Blue Dextran-Sepharose is not susceptible to elution by NADH but can be eluted by high ionic strength. This is presumably due to binding of nitrate reductase to Blue Dextran-Sepharose through a site other than the NADH-binding site.

It is significant, however, that the highest total recovery of nitrate reductase activity was from the column eluted only with KNO_3 (Column 4, Table 9 and Fig. 20). This indicates two things: (a) whereas NADH can only elute a relatively small proportion of the total nitrate reductase activity bound to Blue Dextran-Sepharose, it appears that KNO_3 is capable of eluting (non-specifically) almost all the bound enzyme. Thus KNO_3 can elute nitrate reductase

TABLE 9

ELUTION OF NITRATE REDUCTASE FROM BLUE-DEXTRAN SEPHAROSE

	Column 1	Column 2	Column 3	Column 4
Eluant	5 μM NADH	10 μM NADH	50 μM NADH	0.1 M KNO_3
Total activity Applied (units)*	0.309	0.309	0.309	0.309
Activity Eluted by Eluant (units)*	0.110	0.067	0.096	0.088
Yield with Eluant	35.5%	21.6%	30.9%	28.4%
Activity Eluted by 0.3 M KNO_3 (units)*	0.085	0.074	0.066	0.167
Yield with 0.3 M KNO_3	27.5%	23.8%	21.4%	53.9%
Total Activity Recovered (units)*	0.195	0.141	0.162	0.255
Total Yield	63%	45.5%	52.3%	82.4%

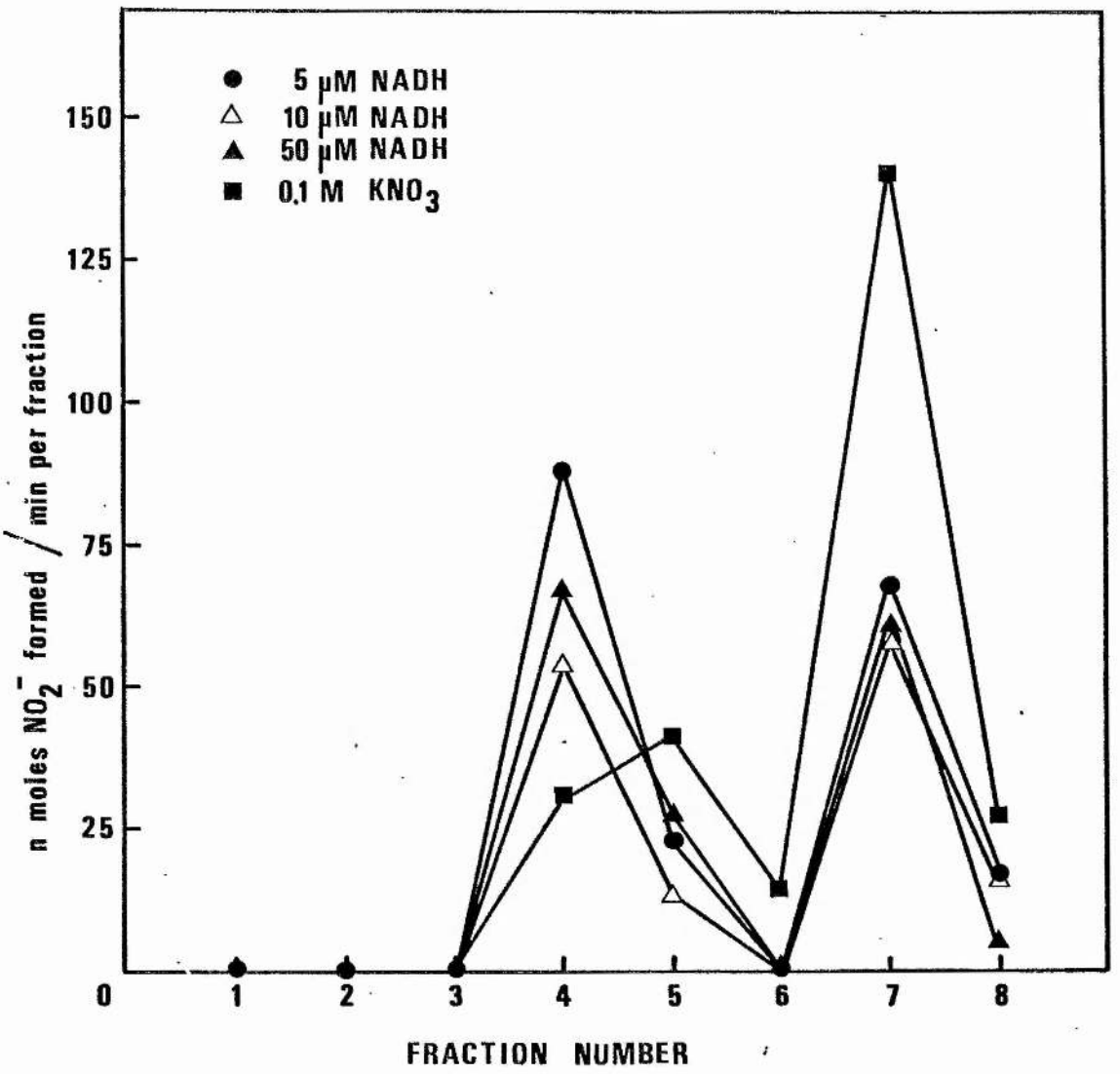
* 1 unit is defined as 1 μmole nitrite produced per minute at 25°C.

FIG. 20

Elution of Nitrate Reductase from Blue Dextran-Sepharose

This figure demonstrates that NADH concentrations as low as 5 μM can elute nitrate reductase from Blue Dextran-Sepharose. Following elution with NADH, further nitrate reductase activity can be recovered by elution of the columns (0.8 cm × 2.5 cm) with 0.3 M KNO_3.

Fractions 1-3 of each column represent sample application and washing with Buffer III to remove non-absorbed protein. Fractions 4 and 5 represent elution with either NADH or 0.1 M KNO_3. Fraction 6 represents elution with Buffer III to remove NADH and KNO_3. Fractions 7 and 8 represent elution wtih 0.3 M KNO_3. Fractions 2-8 were all 10 ml while fraction 1 was 11 ml to allow for the applied sample.


5 µM NADH
10 µM NADH
50 µM NADH
0.1 M KNO3
n moles NO2- formed / min per fraction
150
125
100
75
50
25
0
1
2
3
4
5
6
7
8
FRACTION NUMBER

even if it is bound through its NADH-binding site.
(b) Elution with NADH somehow affects the nitrate reductase resulting in significant levels of inactivation either of enzyme remaining bound to the column or enzyme eluted by the NADH. Thus, elution with KNO_3 after elution with NADH results in total yields 20% lower than those obtained when only KNO_3 is used (compare columns 1 and 4, Table 9).

The results with column 1 (Table 9) also indicate that it should be possible to obtain a 35% yield of highly purified barley nitrate reductase by elution with 5 μM NADH from Blue Dextran-Sepharose. This compares with the yields of 15% obtained in analogous experiments with Blue-Sepharose (Section II).

Purification of Barley Nitrate Reductase with Blue Dextran-Sepharose

In view of the results obtained with column 1 (Table 9) it was decided to attempt to purify barley nitrate reductase by the same method, in the hope of obtaining a 35% yield of active enzyme.

The enzyme sample used for this purification had been derived from 500 g of shoots and had been subjected to streptomycin sulphate treatment, $(NH_4)_2SO_4$ fractionation at 45% saturation, Biogel A1.5 m gel filtration and storage for two months at -70°C in buffer containing 40% glycerol. However, the enzyme sample used had undergone a considerable loss of activity during storage because glycerol

had been added directly to the pooled fractions from Biogel filtration, omitting the $(NH_4)_2SO_4$ concentration step. Hence the protein was stored at a concentration of only 0.3 mg/ml compared with the usual 9-10 mg/ml following concentration with $(NH_4)_2SO_4$. Thus, instead of the usual 80-90% yield of active enzyme after 2 months at -70°C (Section IV) the yield was only 36.5% with the result that the specific activity of the sample, together with the total activity, were much lower than would normally be expected.

As this was the only sample of enzyme available it was applied to a column (2 cm × 9 cm) of Blue Dextran-Sepharose, previously equilibrated with Buffer III, and the column washed with Buffer III until the E_{280} of the washings was zero. Nitrate reductase activity was then eluted with freshly prepared 5 μM NADH in Buffer III and 6 ml fractions were collected throughout (Fig. 21). Fractions 44-48 were pooled, assayed for nitrate reductase activity, and then concentrated by dialysis against polypropylene glycol 2025 at 4°C until the volume was reduced to 4.2 ml, a process which took 5 hours. 0.5 ml of this sample was used to determine the protein concentration. When the 10% TCA was added (see Methods, Section VII) the sample went opaque indicating that some polypropylene glycol had entered the dialysis sac. However, no loss of nitrate reductase activity occurred during the concentration procedure and the contaminating polypropylene glycol was lost from the protein sample during the ethanol washing stage (see Methods,

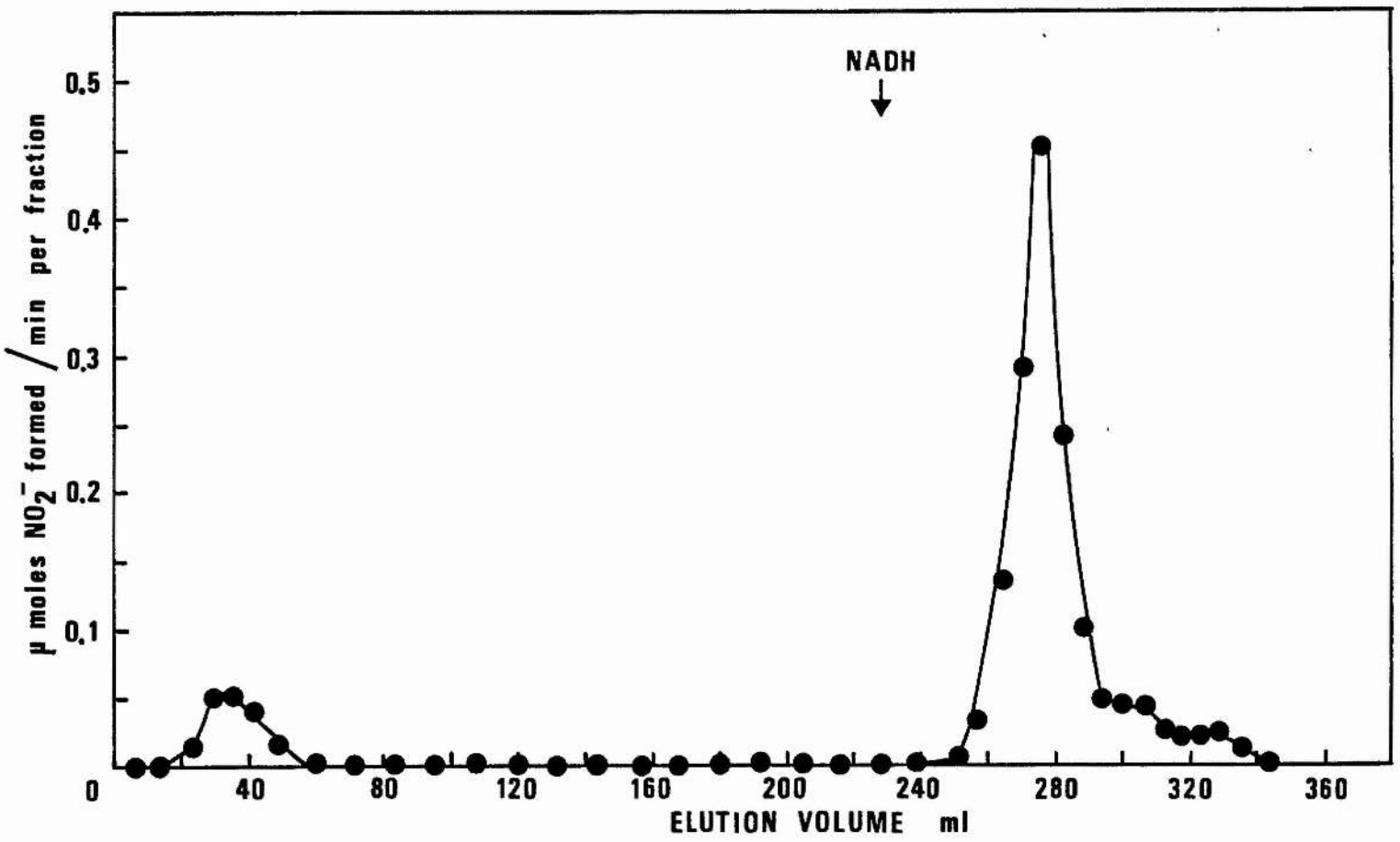

NADH
0.5
0.4
0.3
0.2
0.1
0
μ moles NO_2^- formed /min per fraction
40
80
120
160
200
240
280
320
360
ELUTION VOLUME ml

FIG. 21

Purification of Nitrate Reductase by Blue Dextran-Sepharose Chromatography

This figure shows the elution of nitrate reductase from Blue Dextran-Sepharose with 5 μM NADH. 6 ml fractions were collected from the column (2 cm × 9 cm) and non-absorbed protein was completely removed by elution with Buffer III prior to elution with 5 μM NADH contained in Buffer III.

Section VII). The results of this purification are presented in Table 10 where it can be seen that despite the low specific activity of the applied sample, a very high degree of purification has been achieved.

However, the resultant specific activity of 1.9 units/mg is much lower than would normally be expected for the purification procedure utilised. Reference to Table 10 shows that significant losses in activity were encountered at each of the preliminary purification steps with the result that only 35.8% of the original activity remained following Biogel filtration. Comparison with Table 5 indicates that the yield was normally about 60% by this stage of the purification. As explained above, the sample used for the Blue Dextran-Sepharose purification had also undergone severe losses in activity during storage at -70°C with the result that only 13% of the original activity was applied to Blue Dextran-Sepharose. The expected yield by this stage, based on previous results, is about 50% which is almost 4-fold higher than that encountered in this experiment. As predicted from Table 9 (Column 1) the yield from Blue Dextran-Sepharose was 33.8%. Thus an overall yield of 15-20% is possible for the purification procedure given here. As most of the losses in activity encountered during the early stages would be due to enzyme inactivation, it would appear likely that the amount of protein recovered under conditions of better yield would be comparable with the amount recovered here (Table 10). Thus, not only the yield should be 4-fold

TABLE 10

PURIFICATION OF NITRATE REDUCTASE BY BLUE-DEXTRAN SEPHAROSE CHROMATOGRAPHY

	Nitrate Reductase Activity (units)*	Protein (mg)	Specific Activity (units/mg)	Fold Purification	Yield
Filtered Extract	24.38	6534.9	0.0037	1	100
Streptomycin Sulphate	22.56	4157	0.0054	1.47	92.5
0-45% $(NH_4)_2SO_4$	15.78	1690	0.0093	2.52	64.7
Biogel	8.734	193.4	0.045	12.2	35.8
Storage at -70°C 0-50% $(NH_4)_2SO_4$	3.187	174.3	0.018	4.86	13.1
Blue-Dextran Sepharose	1.079	0.567	1.902	514	4.4

*1 unit is defined as 1 µmole nitrite formed per minute at 25°C.

higher, but also the final specific activity would also be about 4-fold higher, to yield an enzyme sample with specific activity about 8 units/mg. It should also be noted that the final specific activity reported here (Table 10) is an average value for the whole nitrate reductase peak (Fig. 21) indicating that the peak value may in fact be higher.

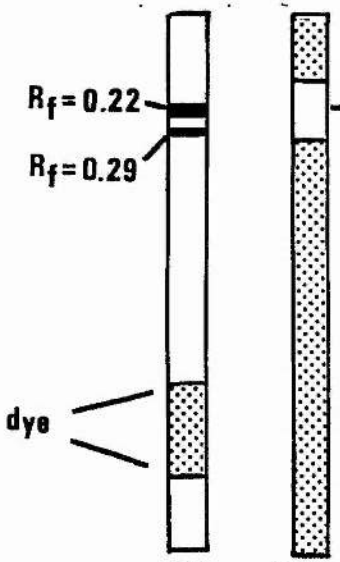
SECTION VII - ELECTROPHORETIC EXAMINATION OF PURIFIED BARLEY NITRATE REDUCTASE

Standard Polyacrylamide Gel Electrophoresis

Following concentration of the purified nitrate reductase by dialysis against polypropylene glycol, 50 µl aliquots were subjected to polyacrylamide gel electrophoresis in 5% acrylamide gels as described in Methods, Section V. Following electrophoresis, the gels were stained for protein, reduced methyl viologen nitrate reductase activity and NADH-dehydrogenase activity. No protein-staining bands were detected as insufficient protein (approximately 5 µg) was applied to the gels. In contrast, both activity stains showed clear results.

Staining for reduced methyl viologen-nitrate reductase activity (see Methods, Section V) revealed one rather broad achromic band of activity, the mid-point of which corresponded at an Rf value of 0.23. (Diagramatic representations of gels following both types of activity staining are given in Fig. 22.) In contrast, two bands of NADH-dehydrogenase activity were detected (Fig. 22) corresponding to Rf values

NADH Dehydrogenase activity



Reduced MV – Nitrate Reductase activity

FIG. 22

Electrophoretic Examination of Purified Nitrate Reductase

This figure shows diagrammatic representations of polyacrylamide gels following electrophoresis of a purified nitrate reductase sample. Gels have been stained (Methods, Section V) for NADH-dehydrogenase activity and reduced methyl viologen (MV) nitrate reductase activity. The diagrams are based on accurate measurements of the gels.

of 0.22 and 0.29. It would seem likely that the slower of these bands corresponds to nitrate reductase which has a very similar Rf value (0.23 compared with 0.22 for NADH-dehydrogenase activity).

It is not possible, however, due to the lack of a protein-stained gel, to ascertain the purity of the nitrate reductase preparation.

SDS Polyacrylamide Gel Electrophoresis of Purified Barley Nitrate Reductase

Aliqots of the purified barley nitrate reductase (Table 10) were subjected to SDS gel electrophoresis by a slight modification of the method of Weber and Osborn (1969), as described in Methods, Section V. Aliquots of ferritin, urease, BSA, catalase and alcohol dehydrogenase were run separately in parallel gels and were used as molecular weight standards. The subunit molecular weights of each of these is given below in Table 11.

TABLE 11

Protein	Subunit Molecular Weight(s)
Ferritin	220 000 18 500
BSA	68 000
Catalase	57 000
Urease	83 000
Alcohol dehydrogenase	41 000

The calibration plot derived from these molecular weight standards by SDS polyacrylamide gel electrophoresis is presented in Fig. 23.

In contrast to the results obtained previously for standard polyacrylamide gel electrophoresis, protein-staining bands were detected after SDS-polyacrylamide gel electrophoresis. The reasons for this are not clear, but it would appear that the protein stain used in these experiments (composition given in Methods, Section V) is much more suited to staining SDS-containing gels than to staining SDS-free gels.

Four major protein-staining bands were detected and the Rf values of these and their corresponding molecular weights are given below in Table 12, together with the values for minor peaks detected by gel scanning.

TABLE 12

	Rf	Molecular Weight
Major peaks	0.025	210 000
	0.116	105 000
	0.195	77 000
	0.298	56 000
Minor peaks	0.248	66 000
	0.399	41 000
	0.458	35 000
	0.505	30 000

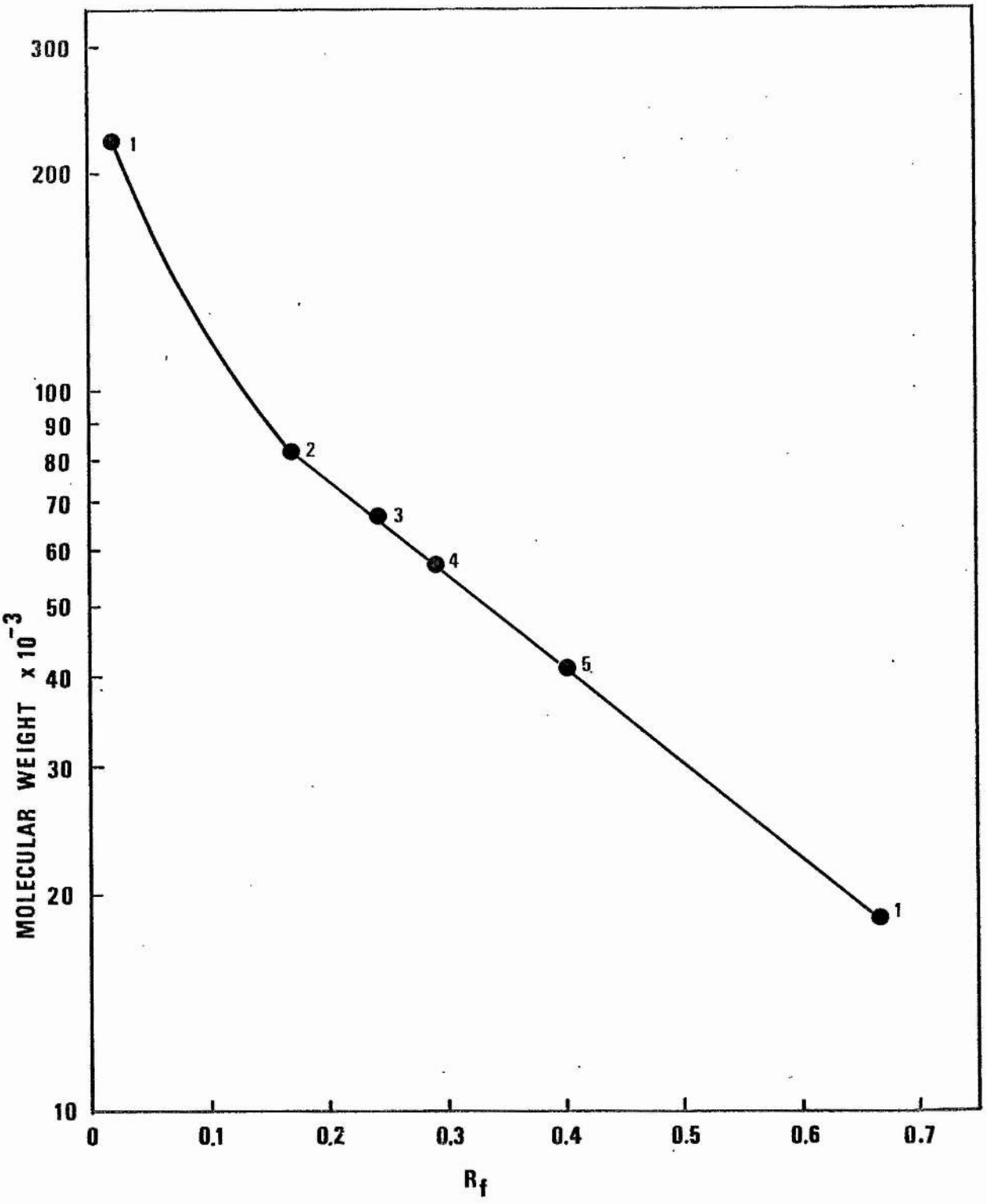
FIG. 23

Molecular Weight Calibration Plot for SDS Gel Electrophoresis

This figure relates the observed R_f values for a number of proteins to their known subunit molecular weights (see Table 11). These proteins are:

1. Ferritin
2. Urease
3. Bovine Serum Albumin
4. Catalase
5. Yeast Alcohol Dehydrogenase.

Electrophoresis was carried out as described in Methods, Section V.


300
200
100
90
80
70
60
50
40
30
20
10
MOLECULAR WEIGHT x 10^{-3}
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
R_f
1
2
3
4
5
1

These results are difficult to interpret as the purity of the applied sample was not known owing to the lack of protein-staining following standard polyacrylamide gel electrophoresis. Consequently, it is not known whether all the protein-staining bands observed after SDS gel electrophoresis are due to nitrate reductase. Possible interpretations of this data will be given in the General Discussion where models for the structure of higher plant nitrate reductase will be presented and examined.

DISCUSSION

In early experiments to purify barley nitrate reductase a surprising variability was found both in the level of nitrate reductase activity extracted and in the yields from preliminary purification steps. It was soon discovered that higher levels of nitrate reductase could be extracted from younger tissue (less than 6 days old) than from older tissue (6-10 days old) but the critical importance of plant age was not recognised. As will be described more fully in Chapter 2 of these Results, and in the General Discussion there is a very marked age-dependent decrease in the stability of barley nitrate reductase. Once this had been recognised, plant age was carefully controlled and shoots were routinely harvested 90 hours after sowing by which time their average height was 4 cm. However, the results presented in Section II of this chapter (experiments with Blue-Sepharose) were obtained with plants older than this. Hence the low initial specific activity reported in Table 3.

The results obtained with Blue-Sepharose were unexpected in that they differed greatly from those of Solomonson (1975) for *Chlorella* nitrate reductase. The elution of 20% of the applied barley nitrate reductase by 0.4 M phosphate buffer (Fig. 5) was confirmed with spinach nitrate reductase by Notton, Fido and Hewitt (1977) who overcame the inability of NADH to elute the remaining nitrate reductase by elution with KCl. However, KCl severely inhibits barley nitrate reductase (Fig. 14) and spinach nitrate reductase is similarly affected (B.A. Notton, personal communication). However, as shown in Fig. 15, much higher yields (approx. 75%) can be obtained by elution with KNO_3 and it is clear that if the Long Ashton workers (Notton, Fido and Hewitt, 1977; Notton *et al.*, 1979) used KNO_3 instead of KCl they would have obtained spinach nitrate reductase at a specific activity of 50 units/mg.

NADH was found to be effective in eluting barley nitrate reductase from Blue Sepharose only if the preliminary washing with 0.4 M phosphate buffer was omitted, but yields of active enzyme were consistently only about 15%. This yield could be markedly increased by the inclusion of low levels of KNO_3 in the buffer during removal of non-absorbed protein from the column and this substrate stabilisation may indicate that either enzyme breakdown or enzyme inactivation may be responsible for the low yields. However, as elution with 0.3 M KNO_3 leads to a 75% recovery of active nitrate reductase (Fig. 15) it would seem unlikely that enzyme breakdown is an important phenomenon.

Work with Blue Dextran-Sepharose instead of Blue-Sepharose revealed some differences between the two methods the first of which was the need to reduce the buffer concentration in order for barley nitrate reductase to bind to Blue Dextran-Sepharose. Secondly, it was found that very low concentrations (5 μM) of NADH could elute barley nitrate reductase from Blue Dextran-Sepharose. Although this concentration was not directly tested with Blue-Sepharose, the results of elution with a gradient of NADH (Fig. 7) indicate that NADH concentrations of 25-30 μM would be necessary to elute barley nitrate reductase from Blue-Sepharose. Thirdly, significantly higher yields of active nitrate reductase were obtained following NADH elution of Blue Dextran-Sepharose (35% yield) than of Blue-Sepharose (15% yield).

Other workers have demonstrated differing responses of proteins to each of these media. Wilson (1976) reported that although Cibacron Blue F3GA appeared to be a universal ligand for nucleotide binding sites, when it was conjugated to form Blue Dextran the binding became much more selective presumably due to steric constraints imposed by the dextran matrix. These constraints are likely to reduce the strength of binding of nitrate reductase and provide a possible explanation for the need for low ionic-strength buffer for use with Blue Dextran-Sepharose.

Further studies on the binding of proteins to Blue-Sepharose and Blue Dextran-Sepharose have been reported by Jankowski *et al.* (1976). They found that fibroblast inter-

feron bound completely to Blue Dextran-Sepharose and could not be displaced even by 1 M NaCl whereas it could be recovered with ethylene glycol which indicates that fibroblast interferon is bound to Blue Dextran-Sepharose by hydrophobic interactions. Binding of fibroblast interferon to Blue-Sepharose was much weaker than to Blue Dextran-Sepharose, but still required ethylene glycol for elution. In contrast, leucocyte interferon could be eluted from Blue Dextran-Sepharose indicating that it was bound electrostatically to the column. With Blue-Sepharose, however, leucocyte interferon would not bind unless the pH was lowered to below its isoelectric point. Nucleotides were unable to elute either interferon from either column.

Thus, the differential effects of Blue-Sepharose and Blue Dextran-Sepharose with barley nitrate reductase are not unexpected. It is also possible to explain some of the yields obtained by analysis of the mechanism of binding of proteins to these affinity media, which can be either electrostatic or hydrophobic.

Comparison of the results obtained with Blue-Sepharose and Blue Dextran-Sepharose shows that elution with only KNO_3 produces very similar high yields (75% and 82% respectively). As KNO_3 is functional through raising the ionic strength, it follows (Jankowski *et al.*, 1976) that 75-82% of the nitrate reductase is bound electrostatically to the columns. This would include the enzyme which can be eluted by NADH, as (for Blue Dextran-Sepharose at least) the

amount of nitrate reductase eluted by NADH (35% of the applied activity) is greater than the amount which is not eluted by KNO_3 (18% of the applied activity).

These results suggest that NADH only elutes the nitrate reductase which is electrostatically bound to the column via the NADH-binding site of the enzyme, whereas KNO_3 can elute all electrostatically-bound enzyme, regardless of binding site. It would therefore seem likely that the differences in yield upon elution of the affinity columns with NADH reflect the extent of binding of nitrate reductase via the NADH-binding site to each of the affinity columns. The ability of ethylene glycol or glycerol (Seelig and Coleman, 1977) to elute nitrate reductase from either column was not examined and so it is not known whether any nitrate reductase is bound through hydrophobic interactions.

It is clear that 0.4 M phosphate buffer was able to elute nitrate reductase from Blue-Sepharose by virtue of raising the ionic strength thereby disrupting the electrostatic binding between nitrate reductase and Blue-Sepharose. It is not clear, however, why only 20% of the applied activity was recovered this way. It has been recently reported (Hewitt, Notton and Garner, 1979) that high buffer concentrations result in loss of MCC from spinach nitrate reductase bound to Blue-Sepharose and it would seem likely, therefore, that this is, at least in part, responsible for the apparent losses in activity.

It is difficult to envisage how the inclusion of low

levels of KNO_3 in the buffers, during the removal of non-absorbed protein from Blue-Sepharose, led to such a marked increase in the level of nitrate reductase eluted by NADH (37% with KNO_3 compared with 15% without) as the KNO_3 was removed prior to elution with NADH. The most likely explanation would appear to be that the KNO_3 bound to the active site of nitrate reductase, on the column, causing a redistribution of charge throughout the enzyme. This in turn would weaken the electrostatic binding of the enzyme to the column such that the increase in ionic strength caused by 100 μM NADH was sufficient to cause elution of the enzyme. The possibility that inclusion of KNO_3 in the buffers for Blue Dextran-Sepharose chromatography may further improve the yield from this medium has not been tested. However, the ability to obtain yields of 35% without such stabilisation makes Blue Dextran-Sepharose much more suitable than Blue-Sepharose for the purification of barley nitrate reductase.

Conclusions and Possible Improvements to the Purification Procedure

Preliminary purification of the enzyme sample prior to affinity chromatography is necessary in order to decrease the amount of applied protein as virtually all nucleotide-requiring proteins will bind to Blue-(Dextran)-Sepharose. Also, as it has not been possible to assess the purity of the nitrate reductase samples it is not known if elution with 5 μM NADH is specific for nitrate

reductase. Thus it may not be possible to purify nitrate reductase by this method unless some preliminary purification is undertaken.

The purification procedures described in Section III permitted the approximately 12-fold purification of nitrate reductase from 250 g of barley shoots to be completed in one day, yielding an enzyme sample which can be stored indefinitely at -70°C. The limiting factor for this is the size of column available, as larger columns would permit the preparation of larger quantities of enzyme. The gel filtration medium used throughout this work was Biogel A1.5 m, 100-200 mesh which combined a sufficiently broad separation range with high flow rates. Recently, however, Pharmacia have marketed an equivalent agarose gel, Sephacryl S-300, which is reported to give better separation characteristics than Biogel A1.5 m, while retaining the same flow rates and retailing at about half the price. It would therefore seem advisable to use Sephacryl S-300 for larger scale columns than those reported here which used 1500 ml of gel.

Another possible modification to the procedures described here would be the inclusion of casein in the extraction buffers in order to protect nitrate reductase from proteolysis (Schrader, Cataldo and Peterson, 1974) and generally increase protein stabilisation. Casein would be preferable to BSA as the addition of BSA to large volumes of extraction buffer would be cost-prohibitive.

It should be possible, therefore, by means of these modifications to obtain at least a 70% yield of nitrate reductase during preliminary purification, combined with the removal of over 90% of the unwanted protein. Thus the sample for application to Blue Dextran-Sepharose would be much cleaner (and smaller) than the corresponding crude extract, and would therefore require a much smaller affinity column which has the added advantage of requiring less time for the removal of non-absorbed protein. The results presented here indicate that a yield of at least 35% can be achieved with Blue Dextran-Sepharose and, if it behaves in a similar manner to Blue-Sepharose, the inclusion of low levels of KNO_3 in the buffers would increase this to well over 50%. Thus, an overall yield of 30-35% for the purification of barley nitrate reductase could be achieved by the methods described here and this would result in markedly increased final specific activities.

CHAPTER 2

ANALYSIS OF NADH-CYTOCHROME c REDUCTASE SPECIES PRESENT IN BARLEY PLANTS

INTRODUCTION

As indicated in the Introduction of this thesis, nitrate reductases have the ability to catalyse other enzymic reactions which are thought to involve only part of the nitrate reductase molecule. One of these partial activities is NAD(P)H-cytochrome c reductase. However, nitrate reductase is not the only source of cytochrome c reductase activity, as was first shown in *N. crassa* by Kinsky and McElroy (1955) who concluded that a second species of NADPH-cytochrome c reductase activity was present in mycelia which had not been exposed to nitrate and therefore lacked nitrate reductase activity. The same conclusions were subsequently reached with *A. nidulans* by Pateman *et al.* (1964).

Separation of the two NADPH-cytochrome c reductase species from *N. crassa* was achieved by Sorger (1966) by sucrose density gradient centrifugation of an extract from nitrate-treated (induced) mycelia. One of these species was a heavy component which was not associated with NADPH-nitrate reductase activity and was equivalent to the major species found in extracts from uninduced *N. crassa* mycelia subjected to sucrose density gradient centrifugation. This component was termed by Sorger (1966) 'the constitutive NADPH-cytochrome c reductase'. The other major NADPH-cytochrome c reductase species found in extracts from induced mycelia was shown to cosediment with NADPH-nitrate

reductase activity and was not present in extracts from uninduced mycelia.

In contrast, analysis of extracts from nitrate-treated barley plants revealed the presence of three NADH-cytochrome c reductase species (Wray and Filner, 1970). The heaviest of these (described as Species A) sedimented to the bottom of sucrose density gradients, was observed in extracts from both nitrate- and nitrate-less plants and is likely to be equivalent to the constitutive NADPH-cytochrome c reductase species found in fungal extracts (Sorger, 1966). It would therefore appear unlikely that this species is related to nitrate reductase.

Nitrate reductase activity was found only in extracts from nitrate plants (Wray and Filner, 1970) and during sucrose density gradient centrifugation it co-sedimented with its partial activities with a sedimentation coefficient of 8S. Identical results had previously been obtained with the nitrate reductase from *N. crassa* by Garrett and Nason (1969). However, in contrast to the results with fungi (Sorger, 1966; Garrett and Nason, 1969; Nason *et al.*, 1974) an additional NADH-cytochrome c reductase species (described as Species C) was found in extracts from barley plants (Wray and Filner, 1970). This species had a sedimentation coefficient of 3.7S and was found in extracts from both nitrate- and nitrate-less plants but the activity associated with this species was approximately 2-fold higher in extracts from nitrate-plants

than from nitrate-less plants. These results were subsequently confirmed by Shen (1972) with extracts from rice shoots.

These observations prompted Wray and Filner (1970) to suggest that the 3.7 S NADH-cytochrome c reductase species was either a dissociation product of nitrate reductase or a precursor protein for that enzyme. If it was a precursor protein, then at least part of the nitrate reductase enzyme is present in nitrate-less plants. The authors did not suggest how this might arise if the 3.7 S species were derived only as a dissociation product of nitrate reductase.

No equivalent NADH-cytochrome c reductase species is found in extracts from wild-type fungal mycelia. However, in the *cnx* mutants of *A. nidulans* and *nit*-1 mutants of *N. crassa*, which lack MCC, a new species is found which has a sedimentation coefficient of 4.5 S (Sorger, 1966; Downey, 1973; Ketchum and Sevilla, 1973). Nason *et al.*, (1974) were able to demonstrate that this 4.5S species was a component of *N. crassa* nitrate reductase by showing that the addition of MCC to an extract from induced *nit*-1 mycelia resulted in the disappearance of the 4.5 S species and the appearance of NADPH-nitrate reductase/-cytochrome c reductase activity with a sedimentation coefficient of 7.8 S.

In this chapter I will describe experiments which were undertaken to determine if the 3.7 S NADH-cytochrome c

reductase species observed in extracts from barley shoots (Wray and Filner, 1970) was related to barley nitrate reductase. Information will also be presented on some physical properties of the NADH-cytochrome c reductase species found in extracts from both nitrate and nitrate-less barley plants.

RESULTS

SECTION I - ANALYSIS OF EXTRACTS FROM NITRATE-LESS PLANTS

By Sephadex G200 Gel Filtration

An extract from nitrate-less plants was prepared as described in Methods Section II, subjected to gel filtration through Sephadex G200 and fractions assayed for NADH-cytochrome c reductase activity (Fig. 24). The first species eluted just after the void volume and probably represents the high molecular weight species which sediments to the bottom of sucrose gradients (Species A in Wray and Filner, 1970). By reference to the internal standards catalase, alcohol dehydrogenase and BSA, and using the correlation of Porath (1963), the Stokes radius of this species was determined to be 9.1 nm.

The other NADH-cytochrome c reductase species was shown to have a Stokes radius of 2.5 nm and is probably equivalent to the low molecular weight species C reported by Wray and Filner (1970).

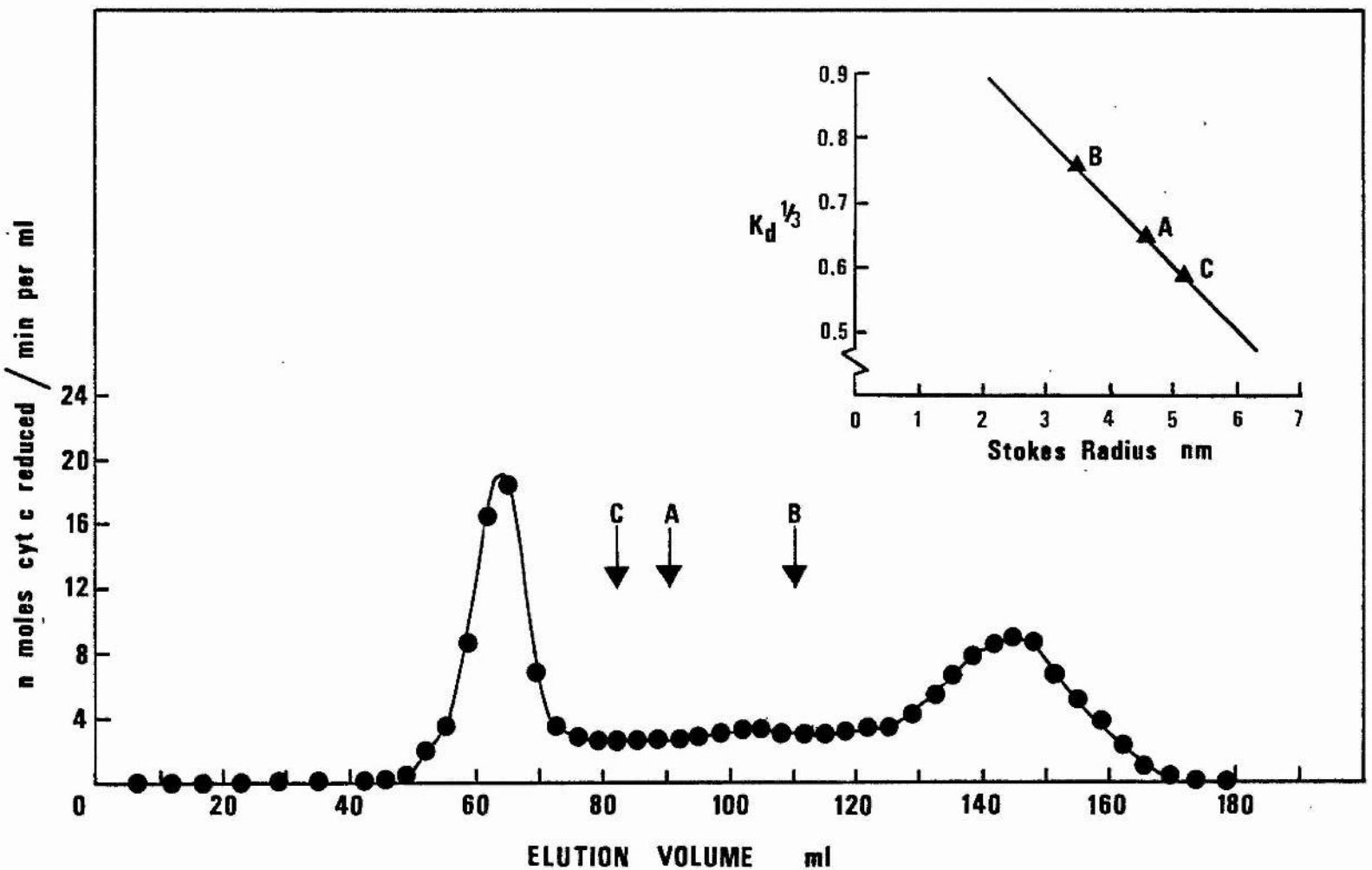

n moles cyt c reduced / min per ml
24
20
16
12
8
4
0
20
40
60
80
100
120
140
160
180
ELUTION VOLUME ml
C
A
B
$K_d^{1/3}$
0.9
0.8
0.7
0.6
0.5
0
1
2
3
4
5
6
7
Stokes Radius nm

FIG. 24

Sephadex G200 Gel Filtration of an Extract from 90-hour old Nitrate-less Plants

This figure shows the distribution of NADH-cytochrome c reductase activity following Sephadex G200 gel filtration of an extract from 90-hour old nitrate-less plants. Two major species of NADH-cytochrome c reductase species are evident, corresponding to Stokes radii of 9.1 nm and 2.5 nm. C, A and B denote the positions of the reference proteins catalase, alcohol dehydrogenase and bovine serum albumin, respectively.

By Sucrose Density Gradient Centrifugation

Extracts from nitrate-less plants were prepared and analysed by sucrose density gradient centrifugation as described in Methods Section IV. The distribution of NADH-cytochrome c reductase activity was determined and revealed the presence of a major species sedimenting in the 3S region (Fig. 25) and probably equivalent to the 3.7 S species C reported by Wray and Filner (1970). The use of larger gradients (18 ml compared to 4.5 ml) and comparison with the three internal standards, catalase, alcohol dehydrogenase and myoglobin, allowed a more accurate measurement of the sedimentation coefficient of this, and other, enzyme species than was achieved by Wray and Filner (1970). From an examination of five experiments the sedimentation coefficient of this NADH-cytochrome c reductase species was determined to be 2.71 ± 0.05 S.

Traces of a second NADH cytochrome c reductase species were detected at the bottom of the gradient and are probably equivalent to the high molecular weight constitutive species A previously mentioned.

Demonstration of Co-identity of the 2.5 nm and 2.7 S NADH-cytochrome c Reductase Species

In order to demonstrate that the 2.5 nm and 2.7 S NADH-cytochrome c reductase species were the same protein a sample from the peak of the 2.5 nm species from Sephadex G200 gel filtration (Fig. 24) was subjected to sucrose

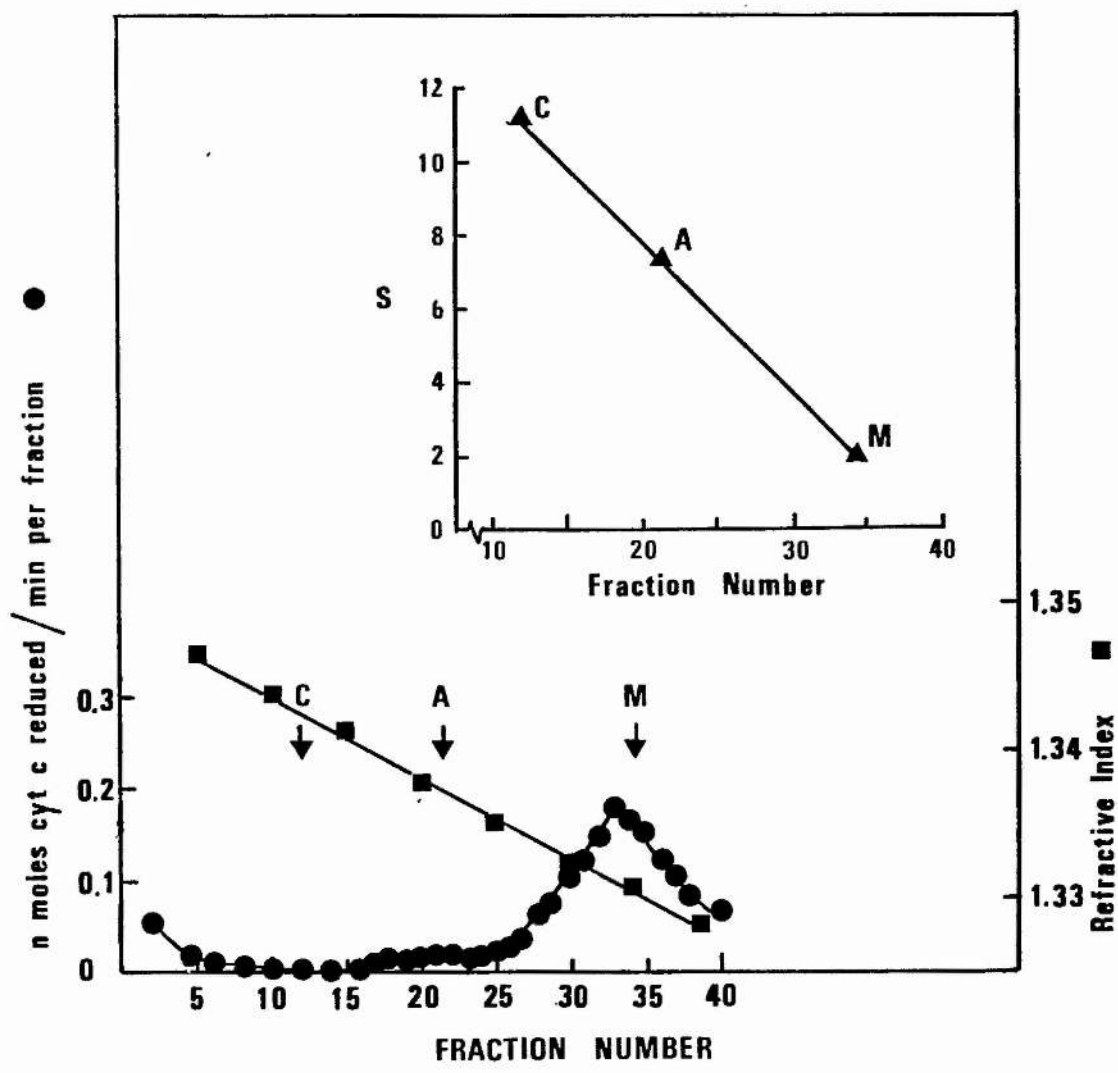

S
12
10
8
6
4
2
0
C
A
M
10
20
30
40
Fraction Number
n moles cyt c reduced / min per fraction
0.3
0.2
0.1
0
C
A
M
5
10
15
20
25
30
35
40
FRACTION NUMBER
1.35
1.34
1.33
Refractive Index

FIG. 25

Sucrose Density Gradient Centrifugation of an Extract from 90-hour old Nitrate-less Plants

This figure shows the distribution of NADH-cytochrome c reductase activity following sucrose density gradient centrifugation of an extract from 90-hour old nitrate-less plants, as described in Methods, Section IV. The major NADH-cytochrome c reductase species has a sedimentation coefficient of 2.7 S. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.

density gradient analysis. However, insufficient activity could be applied to the sucrose gradients to allow its detection after centrifugation. To circumvent this problem a preparative scale gel filtration step was used. For this, 80 g of nitrate-less barley shoots were extracted as described in Methods, Section II, treated with streptomycin sulphate and then centrifuged at 40 000 g for 50 minutes to remove cell debris. The resulting supernatant was adjusted to 80% saturation with $(NH_4)_2SO_4$, precipitated protein collected by centrifugation, dissolved in Buffer II (Buffer I lacking cysteine) and passed through a column of Biogel A1.5 m. (All steps as described in Methods, Section II). The distribution of NADH-cytochrome c reductase activity in the fractions is shown in Fig. 26 where it can be seen that two major NADH-cytochrome c reductase species are again found (compare with Fig. 24) together with trace amounts of some intermediate species.

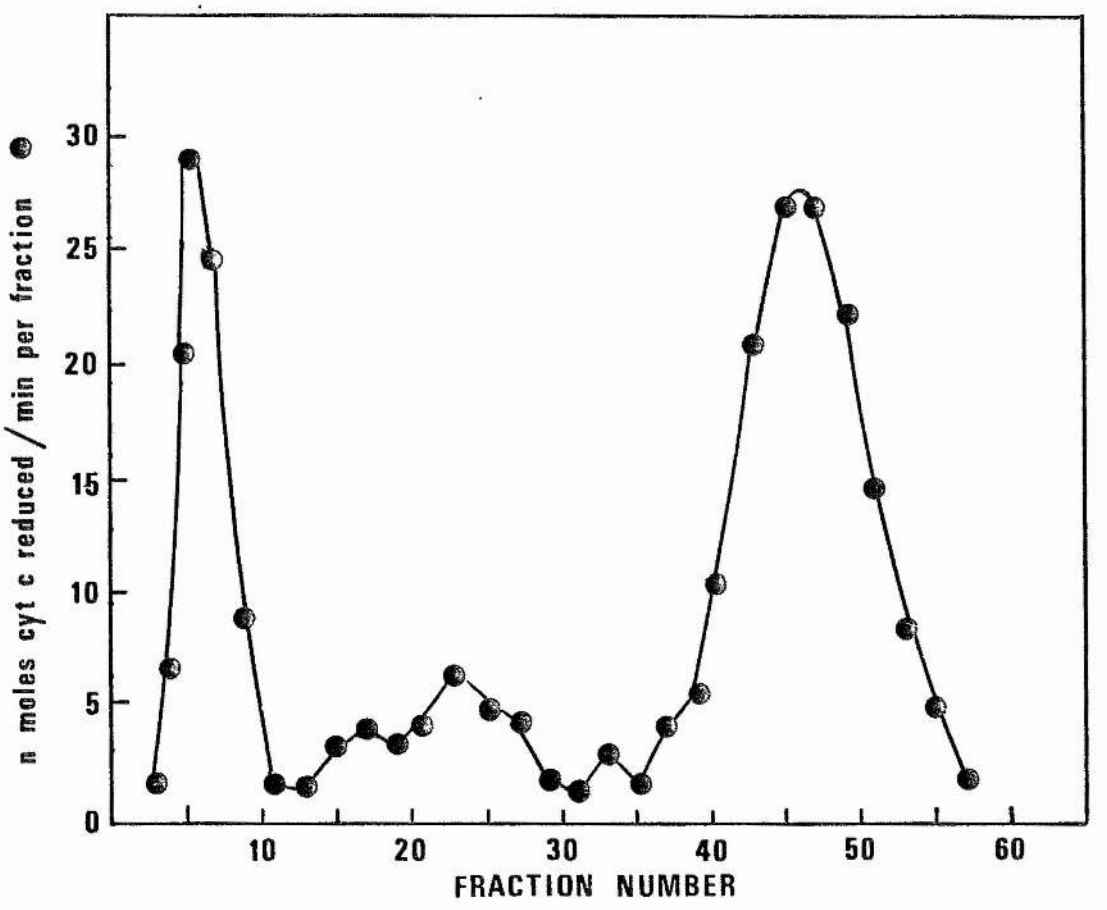
As gel filtration through Sephadex G200 and Biogel A1.5 m separate proteins by the same criteria, namely protein size, it follows that proteins will be eluted in the same order from both columns. As the first-eluted NADH-cytochrome c reductase species is eluted at the void volume from both gels it follows that the only other major species found by these methods are the same protein.

Fractions from the trailing edge of the second-eluted major species from Biogel A1.5 m (equivalent to the 2.5 nm species from Sephadex G200) were then subjected to sucrose

FIG. 26

Biogel Filtration of an Extract from 90-hour Old Nitrate-less Plants

This figure shows the distribution of NADH cytochrome c reductase activity following gel filtration of an extract from 90-hour old nitrate-less plants through Biogel A1.5 m.


n moles cyt c reduced / min per fraction
0
5
10
15
20
25
30
10
20
30
40
50
60
FRACTION NUMBER

density gradient analysis and the major NADH-cytochrome c reductase species was found to sediment at 2.7 S (Fig. 27). It is therefore likely that the small NADH-cytochrome c reductase species found after Biogel filtration (Fig. 26) is equivalent to the 2.5 nm species found after Sephadex G200 gel filtration (Fig. 24) and is the same species as that found sedimenting at 2.7 S after sucrose density gradient analysis.

Calculation of Molecular Parameters of NADH-Cytochrome c Reductase Species found in Extracts from Nitrate-less Plants

The low molecular weight NADH-cytochrome c reductase found in nitrate-less plants has a Stokes radius of 2.5 nm and a sedimentation coefficient of 2.7 S which gives a calculated molecular weight of 27 800 daltons. This protein has a frictional ratio of 1.25, an axial ratio of 4:1 and thus is relatively small and globular.

The larger NADH cytochrome c reductase species (equivalent to Species A in Wray and Filner (1970) and to the constitutive species in Sorger, 1966) is unlikely to be related to nitrate reductase and was not further studied.

SECTION II - ANALYSIS OF EXTRACTS FROM NITRATE PLANTS

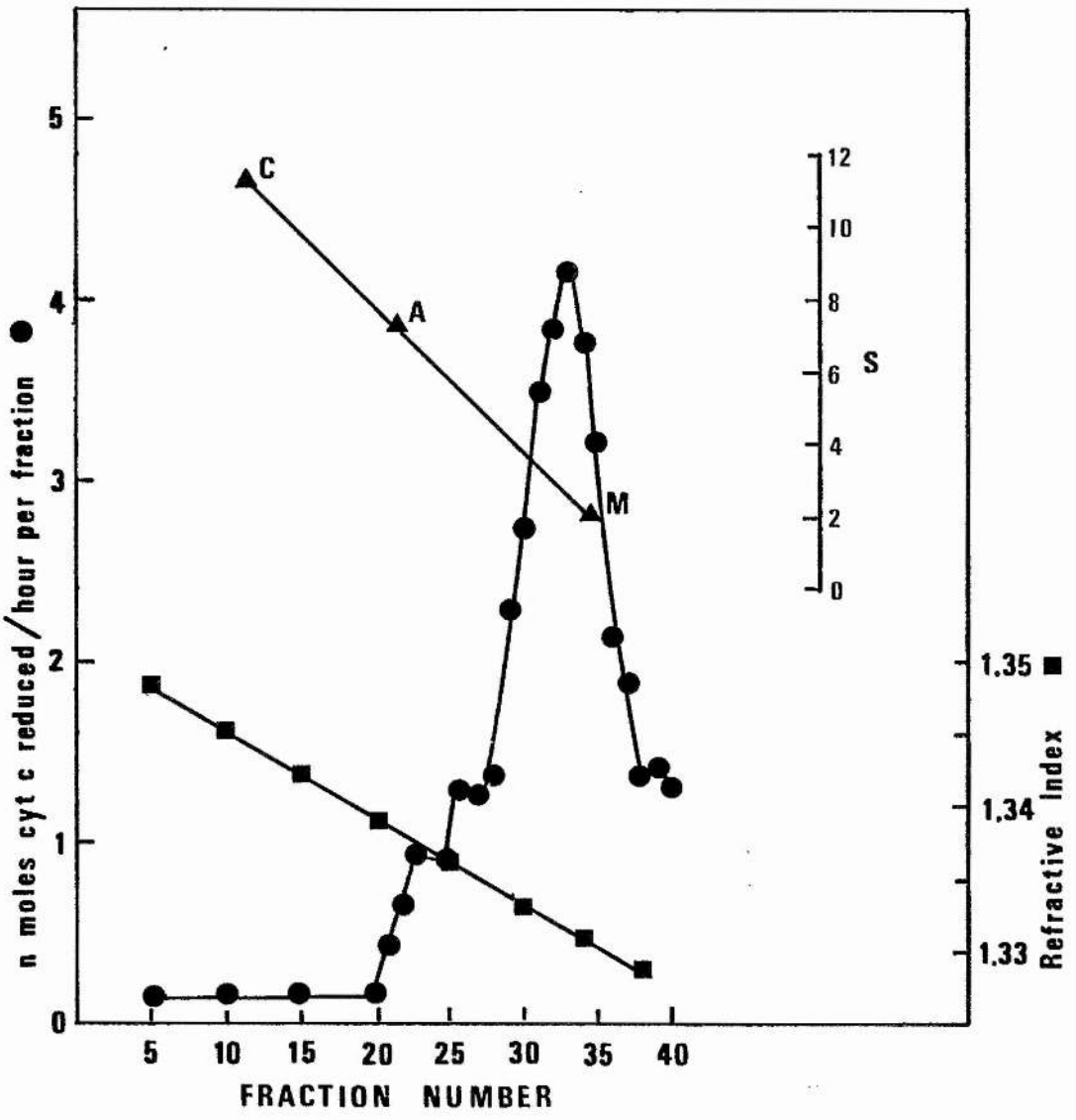
By Sucrose Density Gradient Centrifugation

An extract from nitrate-treated plants was prepared and subjected to sucrose density gradient centrifugation and the resultant distribution of NADH-cytochrome c reductase

FIG. 27

Sucrose Density Gradient Centrifugation Analysis of Fraction 51 (Fig. 26)

This figure shows the distribution of NADH-cytochrome c reductase activity following sucrose density gradient centrifugation of an aliquot from fraction 51 (Fig. 26). C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


n moles cyt c reduced/hour per fraction ●
0
1
2
3
4
5
C
A
M
12
10
8
6
4
2
0
S
1.35 ■
1.34
1.33
Refractive Index
5
10
15
20
25
30
35
40
FRACTION NUMBER

activity determined (Fig. 28). Trace amounts of a high molecular weight species were detected at the bottom of the gradient, as was found after analysis of nitrate-less plant extracts (Fig. 25).

The major species of NADH-cytochrome c reductase in the extract was found to co-sediment with NADH-nitrate reductase activity, FMNH-nitrate reductase activity (data not shown) and reduced methyl viologen nitrate reductase activity (Fig. 28) and is equivalent to species B reported by Wray and Filner (1970). The third NADH-cytochrome c reductase species seen in Fig. 28 has a sedimentation coefficient of ca. 3 S and is equivalent to species C reported by Wray and Filner (1970).

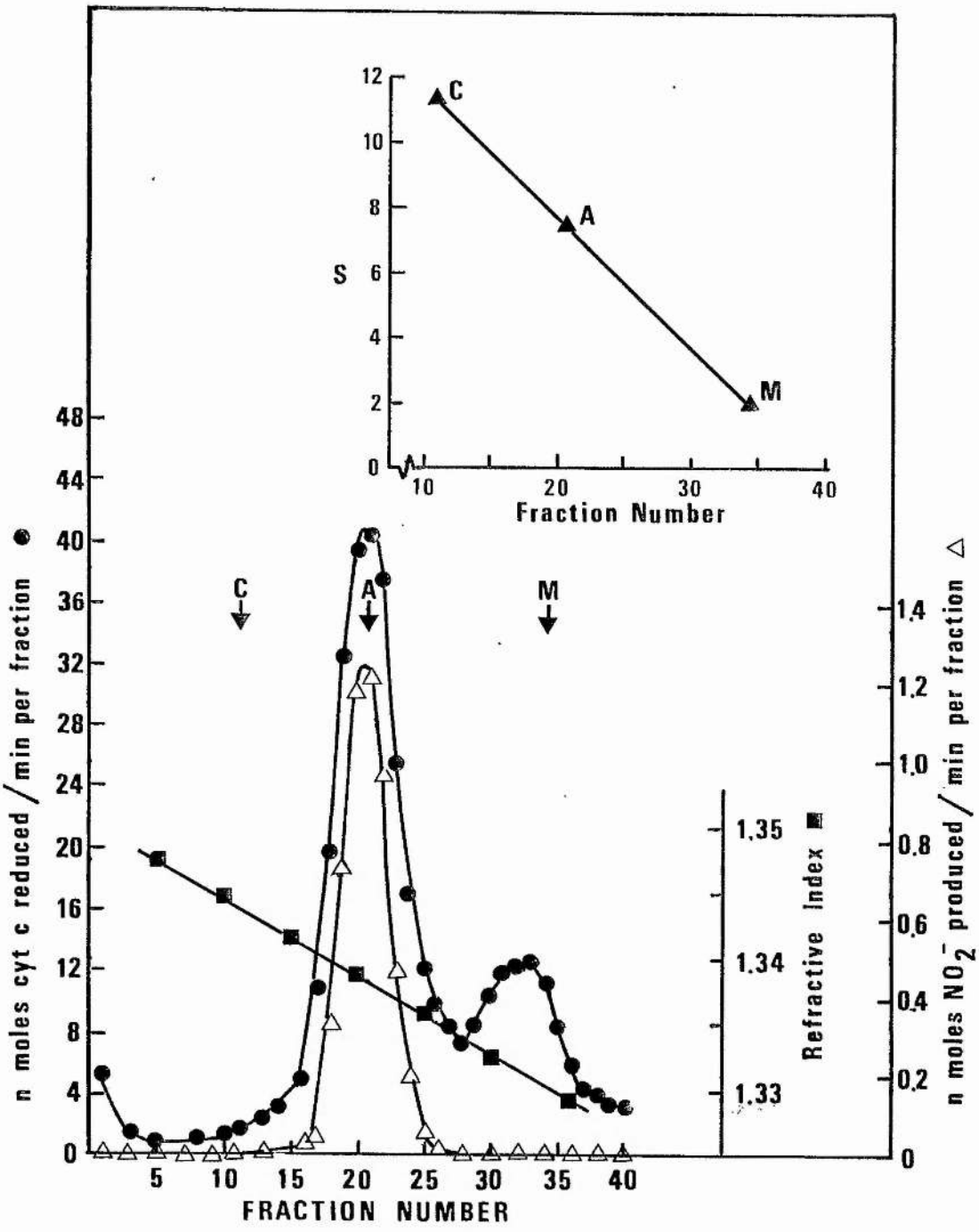
From an examination of eleven experiments the sedimentation coefficient of the nitrate-reductase associated NADH-cytochrome c reductase species was found to be 7.69 ± 0.06 S. A symmetrical distribution of activity was consistently found for this peak provided that the plants used were not significantly older than 90 hours (see later).

In contrast, the ca. 3 S NADH-cytochrome c reductase species exhibited a variable profile with a larger range of sedimentation coefficient. An examination of nine experiments showed that the sedimentation coefficient of this species was 3.05 ± 0.10 S. The activity profile exhibited by this species differed in two important ways

FIG. 28

Sucrose Density Gradient Centrifugation of an Extract from 90-hour old Nitrate Plants

This figure shows the distribution of NADH-cytochrome c reductase and reduced methyl viologen-nitrate reductase activities following sucrose density gradient centrifugation of an extract from 90-hour old nitrate plants. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


S
Fraction Number
C
A
M
n moles cyt c reduced / min per fraction ●
n moles NO_2^- produced / min per fraction △
Refractive Index ■
FRACTION NUMBER

from that found in nitrate-less plants (Fig. 25). Firstly, the level of activity found in the ca. 3 S region in nitrate plant extracts was approximately twice that found in the same region for extracts from nitrate-less plants, as was previously reported by Wray and Filner (1970). Secondly, the sedimentation coefficient of 2.71 S determined for the NADH-cytochrome c reductase from nitrate-less plants was lower than the lowest value found for the sedimentation coefficient of the NADH-cytochrome c reductase from nitrate plants (range 2.95 - 3.15 S).

These results suggest that the NADH-cytochrome c reductase species seen in the ca. 3 S region in extracts from nitrate-less plants is not the same as the NADH-cytochrome c reductase species seen in the ca. 3 S region from nitrate-plants. Extracts from nitrate-less plants possess a single small NADH-cytochrome c reductase species which sediments at 2.71 S. The variable ca. 3 S profile seen in extracts from nitrate plants may be explained by the presence of other NADH-cytochrome c reductase species, somewhat heavier than 2.71 S, in addition perhaps to the 2.71 S species. The activity profile in the ca. 3 S region seen in extracts from nitrate plants would then be the result of the summation of the activity profiles of individual NADH-cytochrome c reductase species possessing closely similar sedimentation coefficients.

By Sephadex G 200 Gel Filtration

Analysis of extracts from nitrate plants by Sephadex

G200 gel filtration revealed the presence of several NADH-cytochrome c reductase species (Fig. 29) the major one of which co-eluted with NADH-nitrate reductase activity and had a Stokes radius of 6.4 nm. The shoulder on the leading edge of this peak is equivalent to the first-eluted NADH-cytochrome c reductase species in nitrate-less plants (Fig. 24) since in other experiments where it was eluted as a discrete peak it was shown to have a Stokes radius of 9.1 nm.

Three smaller NADH-cytochrome c reductase species were eluted at 102 ml, 118 ml and 142 ml (Fig. 29). These species were consistently observed to be present in extracts from nitrate plants and Stokes radii of 3.9 nm, 3.1 nm and 2.5 nm have been assigned to them although they were present in very small amounts (especially the 3.9 nm species) and did not elute as discrete peaks. The 2.5 nm species was also found in extracts from nitrate-less plants (Fig. 24) and when the data from Figs. 24 and 29 are plotted on the same scale (Fig. 30) it is evident that the level of the 2.5 nm NADH-cytochrome c reductase does not change upon exposure of the plants to nitrate. Thus it can be concluded that the change in sedimentation coefficient of the NADH-cytochrome c reductase activity in the ca. 3 S region of sucrose density gradients upon exposure of the plants to nitrate (Figs. 25 and 28) is due to the appearance of at least two new NADH-cytochrome c reductase species, having Stokes radii of 3.9 nm and 3.1 nm respectively. These species have not previously been reported in the literature.

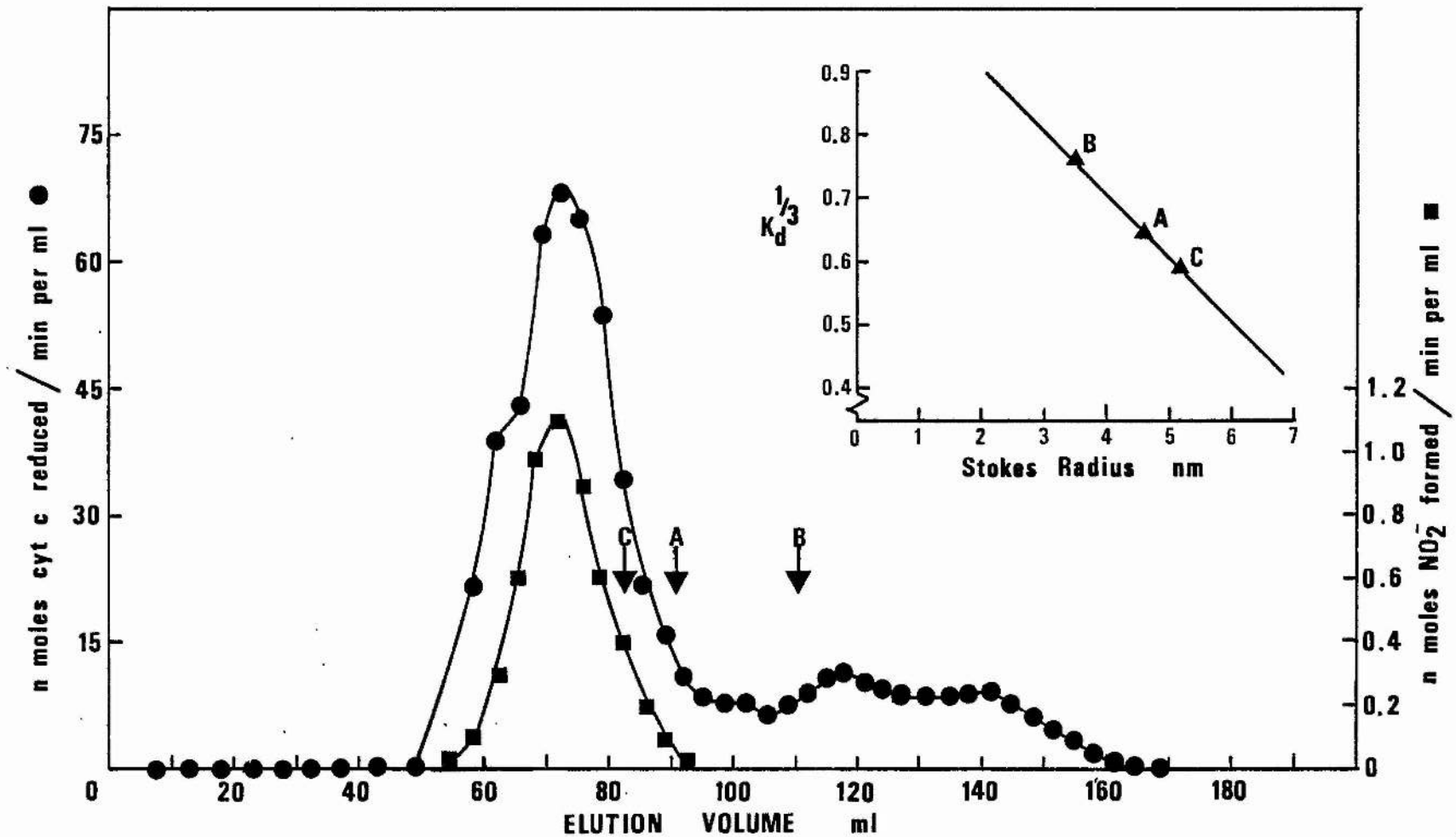

n moles cyt c reduced / min per ml ●
n moles NO_2^- formed / min per ml ■
ELUTION VOLUME ml
0
20
40
60
80
100
120
140
160
180
15
30
45
60
75
0.2
0.4
0.6
0.8
1.0
1.2
A
B
C
$K_d^{1/3}$
0.4
0.5
0.6
0.7
0.8
0.9
Stokes Radius nm
1
2
3
4
5
6
7

FIG. 29

Sephadex G200 Gel Filtration of an Extract from 90-hour old Nitrate Plants

This figure shows the distribution of NADH-cytochrome c reductase and NADH-nitrate reductase activities following Sephadex G200 gel filtration of an extract from 90-hour old nitrate plants. C, A and B denote the positions of the reference proteins catalase, alcohol dehydrogenase and bovine serum albumin, respectively.

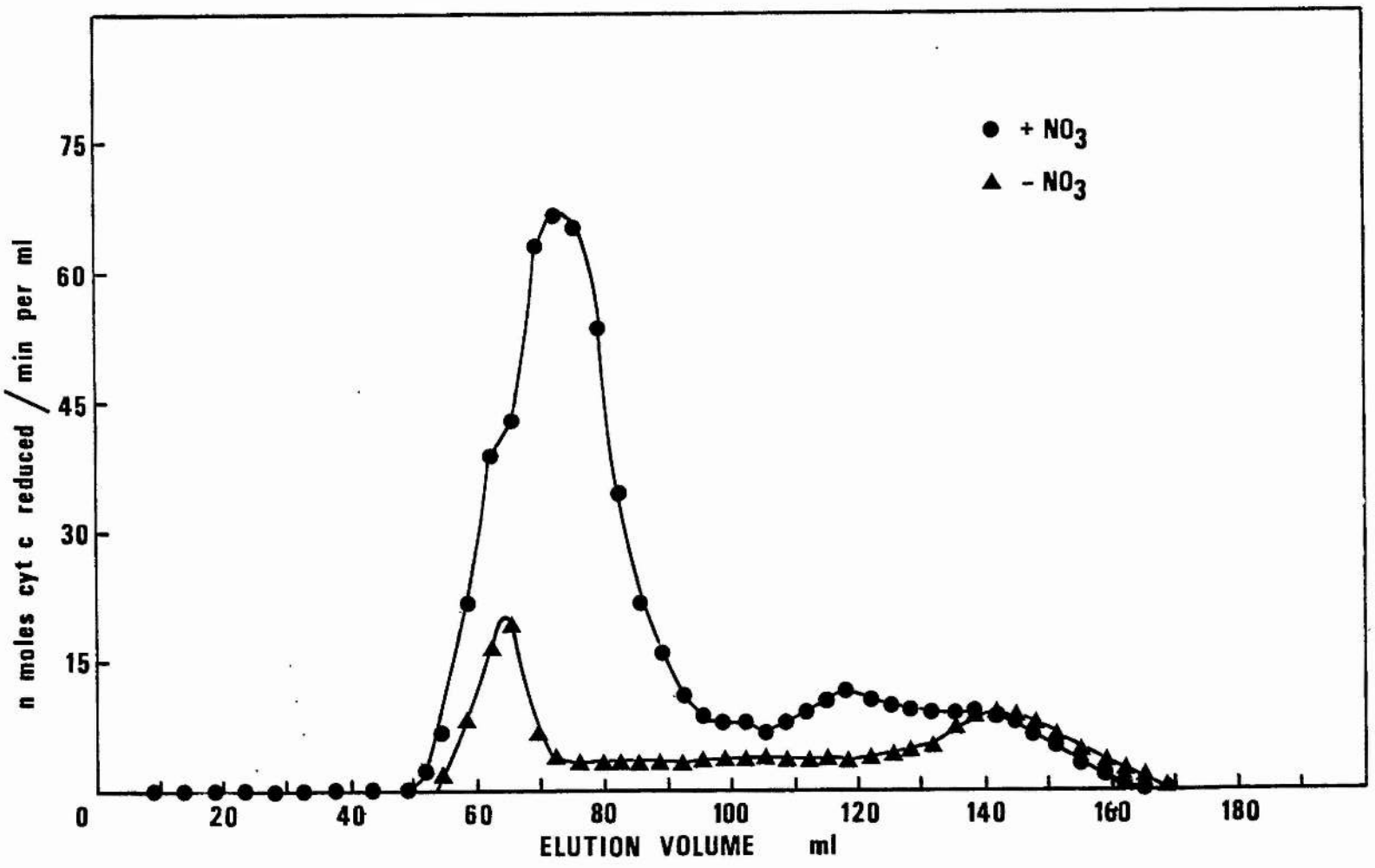

+ NO3
- NO3
n moles cyt c reduced / min per ml
75
60
45
30
15
0
20
40
60
80
100
120
140
160
180
ELUTION VOLUME ml

FIG. 30

Sephadex G200 Gel Filtration of Extracts from 90-hour Old Nitrate-less and Nitrate-Plants

This figure combines the data presented in Figs. 24 and 29 and shows the distribution of NADH-cytochrome c reductase activity from both nitrate-less (Fig. 24) and nitrate-plants (Fig. 29). Both sets of data are plotted here on the same scale to allow direct comparison of the two profiles.

Evidence that the New NADH Cytochrome c Reductase Species are Derived from Nitrate Reductase

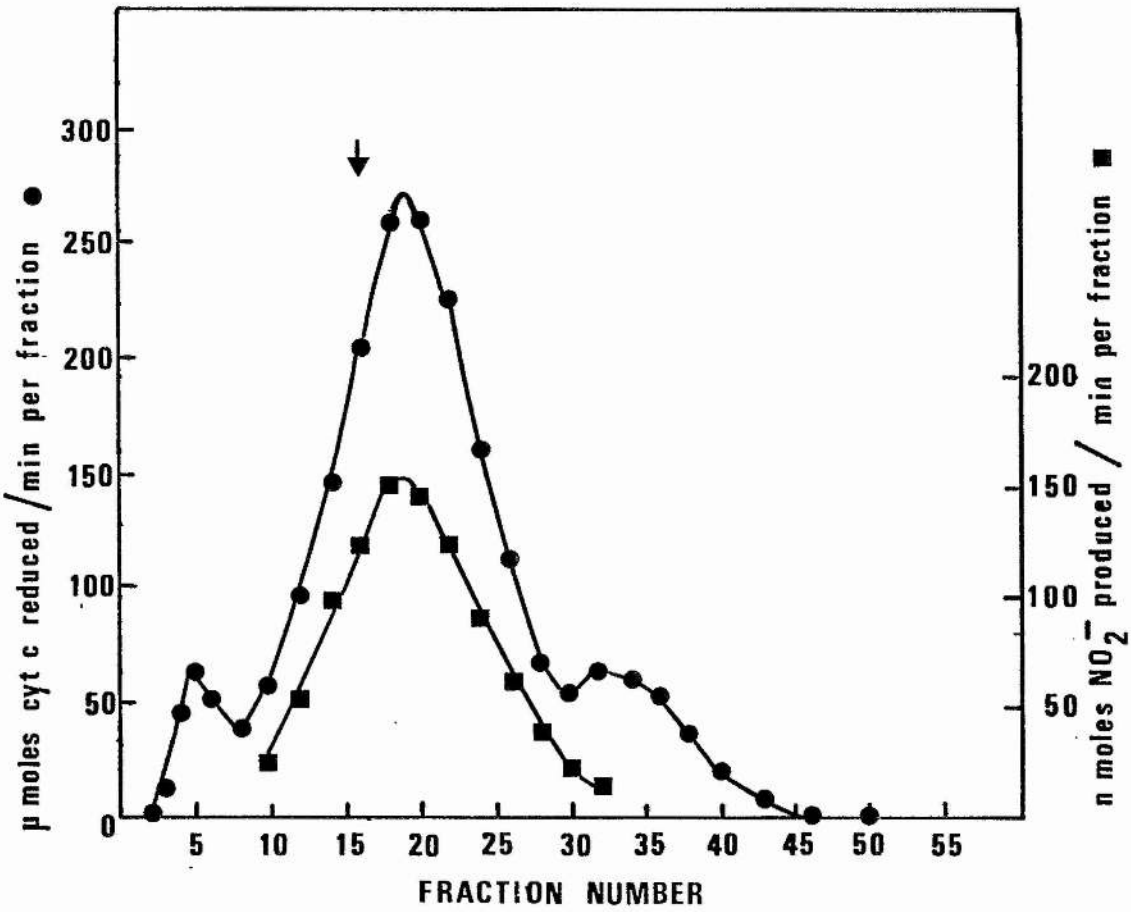
Since the 3.9 nm and 3.1 nm NADH-cytochrome c reductase species are present only in extracts from nitrate plants it is likely that they are related to nitrate reductase which itself is only present in extracts from nitrate plants and which possesses NADH-cytochrome c reductase activity (Figs. 29 and 30). These species could be newly synthesised nitrate reductase precursor subunits destined ultimately for assembly into functional nitrate reductase, or they could be derived from nitrate reductase. In an attempt to see if these species were derived from nitrate reductase a sample from the leading edge of the nitrate reductase peak obtained after Sephadex G200 gel filtration analysis (Fig. 29) was subjected to sucrose density gradient centrifugation. However, as was the case in Section I, insufficient activity could be applied to the gradients to allow subsequent analysis. This problem was circumvented, as before, by performing the initial step on a preparative scale.

150 g of nitrate-treated barley shoots were extracted as described in Methods, Section II and the protein precipitating between 0 and 60% saturation with $(NH_4)_2SO_4$ was collected by centrifugation, dissolved in Buffer II, subjected to gel filtration through Biogel A1.5 m and the resultant distribution of NADH-cytochrome c reductase activity determined (Fig. 31). The first-eluted NADH-

FIG. 31

Biogel Filtration of an Extract from 90-hour Old Nitrate Plants

This figure shows the distribution of NADH-cytochrome c reductase and NADH-nitrate reductase activities following gel filtration of an extract from 90-hour plants through Biogel A1.5 m. The arrow denotes fraction 16 which was analysed by sucrose density gradient centrifugation (Fig. 32) and Sephadex G200 gel filtration (Fig. 33).


300
250
200
150
100
50
0
5
10
15
20
25
30
35
40
45
50
55
FRACTION NUMBER
μ moles cyt c reduced /min per fraction ●
n moles NO_2^- produced / min per fraction ■
200
150
100
50

cytochrome c reductase species again elutes just after the void volume and is probably equivalent to the 9.1 nm species detected after Sephadex G200 gel filtration (Fig. 30). The second-eluted species co-eluted with nitrate-reductase and represents a partial activity of that enzyme. The last eluted species is likely to be a mixture of the 3.9 nm and 3.1 nm species, the 2.5 nm species not having been precipitated by 60% $(NH_4)_2SO_4$ (see later).

Sucrose density gradient analysis of fraction 16 taken from the leading edge of the major NADH-cytochrome c reductase peak, in order to minimise contamination with smaller species, produced the profile of NADH-cytochrome c reductase activity seen in Fig. 32. In addition to the major species, which sediments at 7.7 S, there is an additional heterogeneous peak which appears to consist of two NADH-cytochrome c reductase species to which sedimentation coefficients of 3.8 S and 3.1 S have been assigned. The amount of these species relative to the 7.7 S species is much greater than could be accounted for by contamination of fraction 16 (Fig. 31) and it can thus be concluded that the 3.8 S and 3.1 S NADH-cytochrome c reductase species are derived from nitrate reductase.

Sephadex G200 gel filtration analysis (Fig. 33) of fraction 16 (Fig. 31) gave rise to one major NADH-cytochrome c reductase species with a Stokes radius of 6.4 nm plus two additional species eluting at 102 ml and 118 ml and which possess Stokes radii of 3.9 nm and 3.1 nm respectively.

FIG. 32

Sucrose Density Gradient Centrifugation Analysis of Fraction 16 (Fig. 31)

This figure shows the distribution of NADH-cytochrome c reductase activity following sucrose density gradient centrifugation of an aliquot from fraction 16 (Fig. 31) C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.

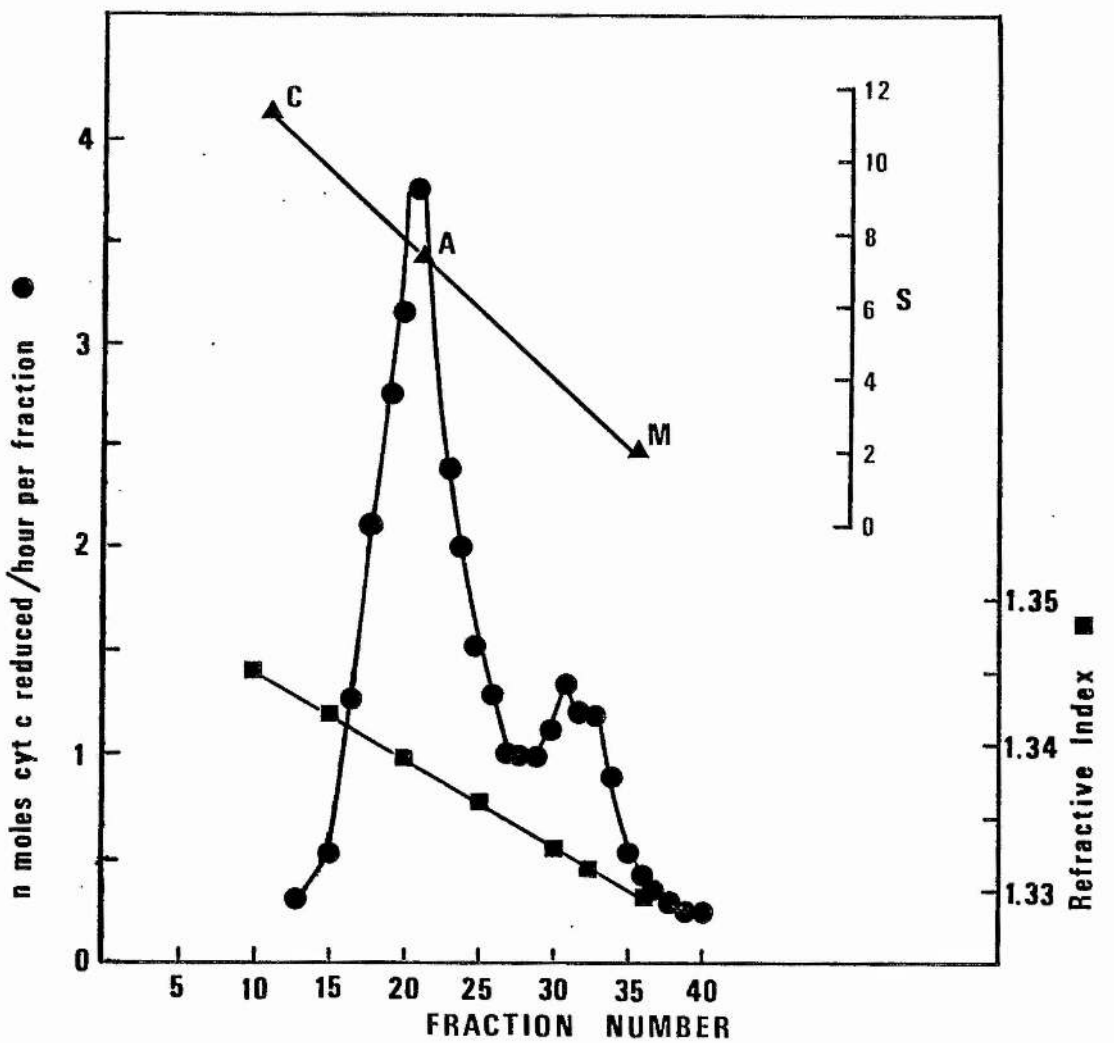
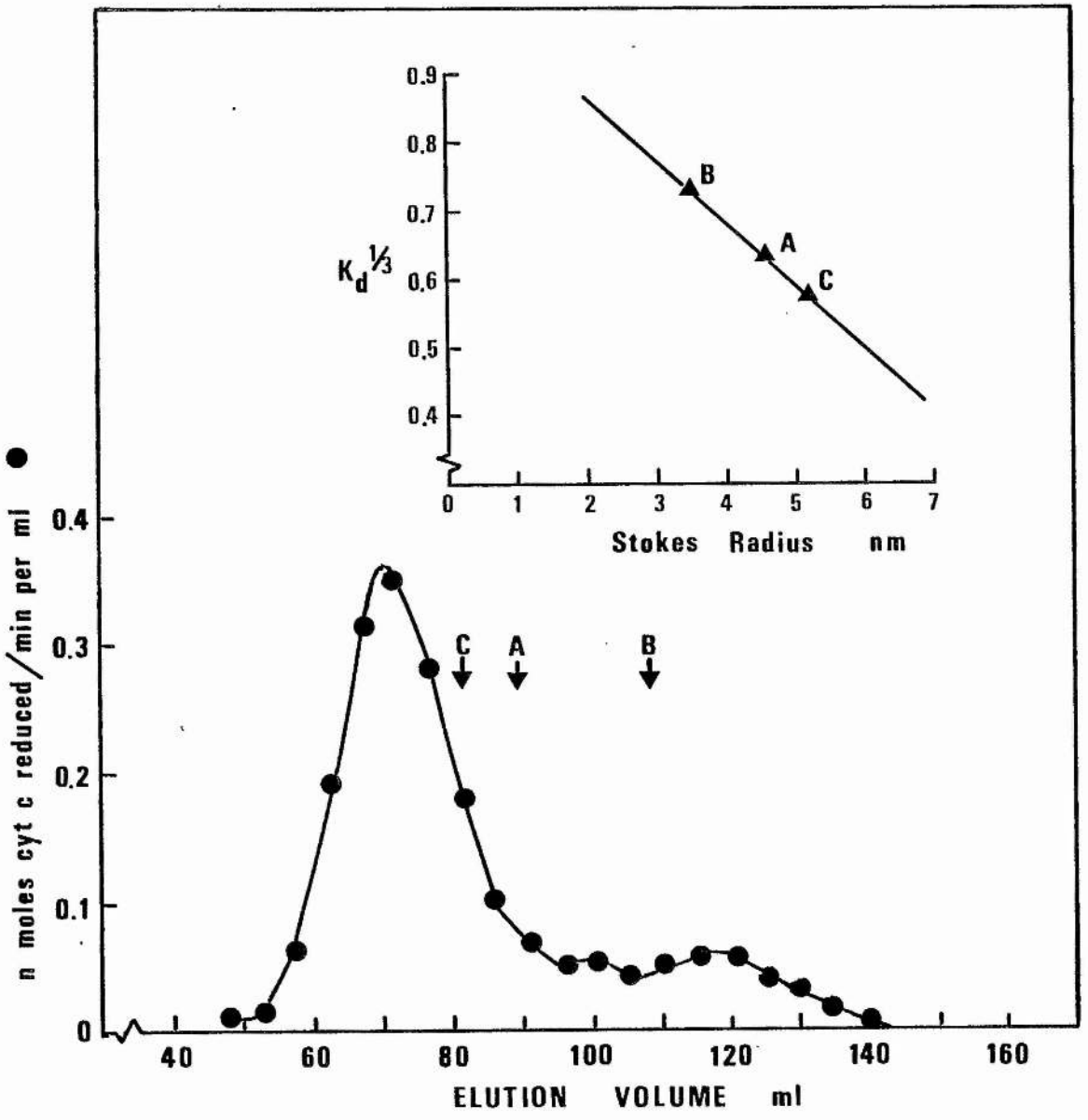

C
A
M
S
n moles cyt c reduced /hour per fraction ●
Refractive Index ■
FRACTION NUMBER
0
1
2
3
4
5
10
15
20
25
30
35
40
0
2
4
6
8
10
12
1.33
1.34
1.35

FIG. 33

Sephadex G200 Gel Filtration Analysis of Fraction 16 (Fig. 31)

This figure shows the distribution of NADH-cytochrome c reductase activity following gel filtration of an aliquot from fraction 16 (Fig. 31) through Sephadex G200. C, A and B denote the positions of the reference proteins catalase, alcohol dehydrogenase and bovine serum albumin, respectively.


0.9
0.8
0.7
$K_d^{1/3}$
0.6
0.5
0.4
B
A
C
0 1 2 3 4 5 6 7
Stokes Radius nm
n moles cyt c reduced/min per ml
0.4
0.3
0.2
0.1
0
C
A
B
40 60 80 100 120 140 160
ELUTION VOLUME ml

These values are the same as those possessed by the two small NADH-cytochrome c reductase species which are present only in extracts from nitrate plants (Fig. 30).

It may be concluded therefore that at least part, and perhaps all, of the 3.9 nm and 3.1 nm NADH-cytochrome c reductase species present in nitrate plant extracts are derived from nitrate reductase. It may also be concluded that the 27 800 molecular weight NADH-cytochrome c reductase, which is present both in nitrate- and nitrate-less plant extracts, is probably not related to nitrate reductase.

SECTION III - EFFECT OF PLANT AGE ON THE TYPES OF NADH-CYTOCHROME c REDUCTASE SPECIES PRESENT IN EXTRACTS FROM NITRATE PLANTS

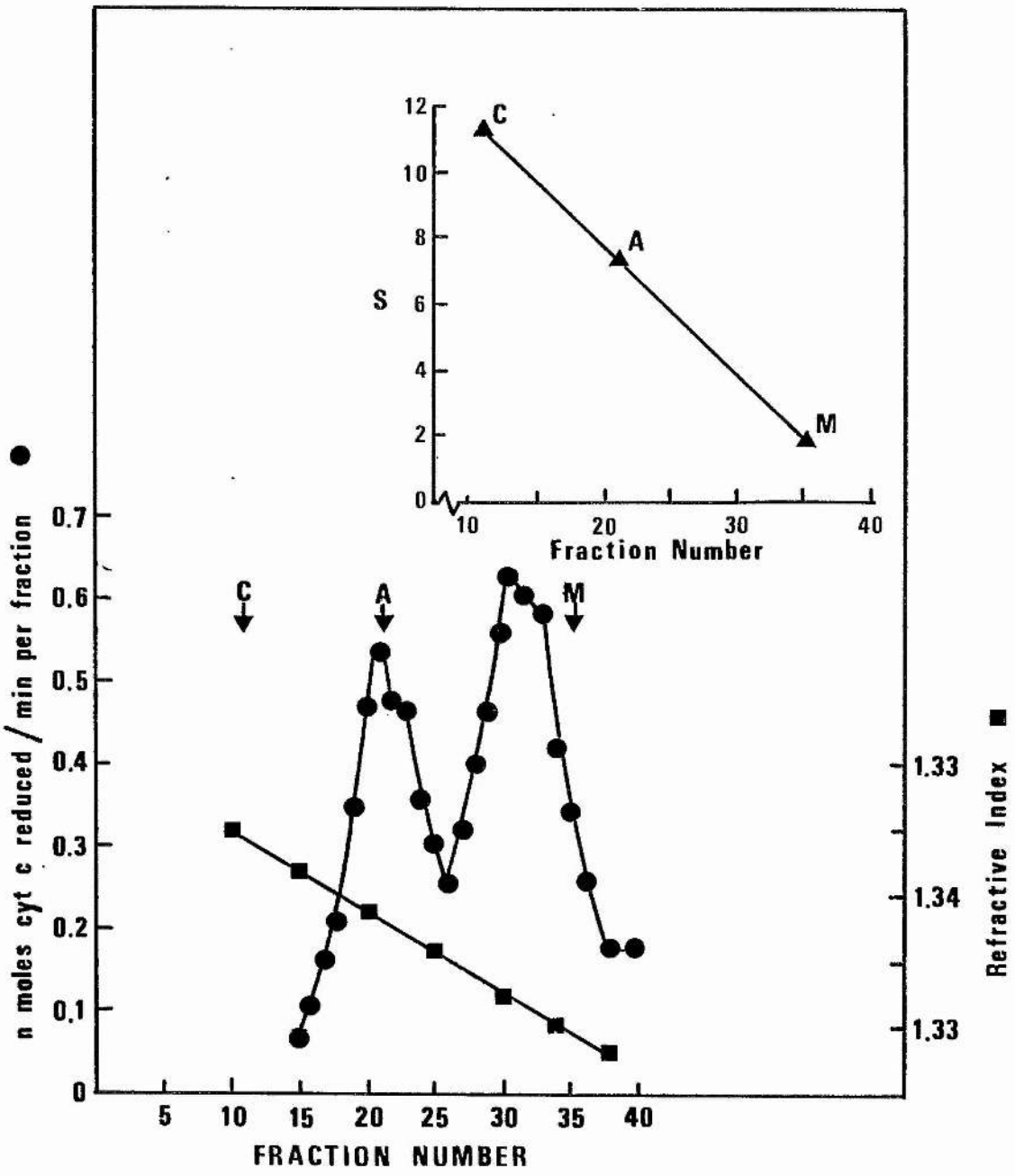
As indicated earlier the patterns of NADH-cytochrome c reductase described so far are only found in extracts from barley plants which are no older than 90 hours, with a shoot length of 4-5 cm. The results to be presented in this section were obtained some time before their correlation with plant age was understood. This relationship has been elucidated in this laboratory by J. Brown (unpublished observations) and the results presented here are given to illustrate the relevent phenomena, as these were utilised in later work.

Sucrose density gradient analysis of extracts of 120-hour old nitrate plants (Fig. 34) resulted in a profile of

FIG. 34

Sucrose Density Gradient Centrifugation of an Extract from 120-hour Old Nitrate-plants

This figure shows the distribution of NADH-cytochrome c reductase activity following sucrose density gradient centrifugation of an extract from 120-hour old nitrate plants. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


S
12
10
8
6
4
2
0
C
A
M
10
20
30
40
Fraction Number
n moles cyt c reduced / min per fraction ●
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C
A
M
5
10
15
20
25
30
35
40
FRACTION NUMBER
1.33
1.34
1.33
Refractive Index ■

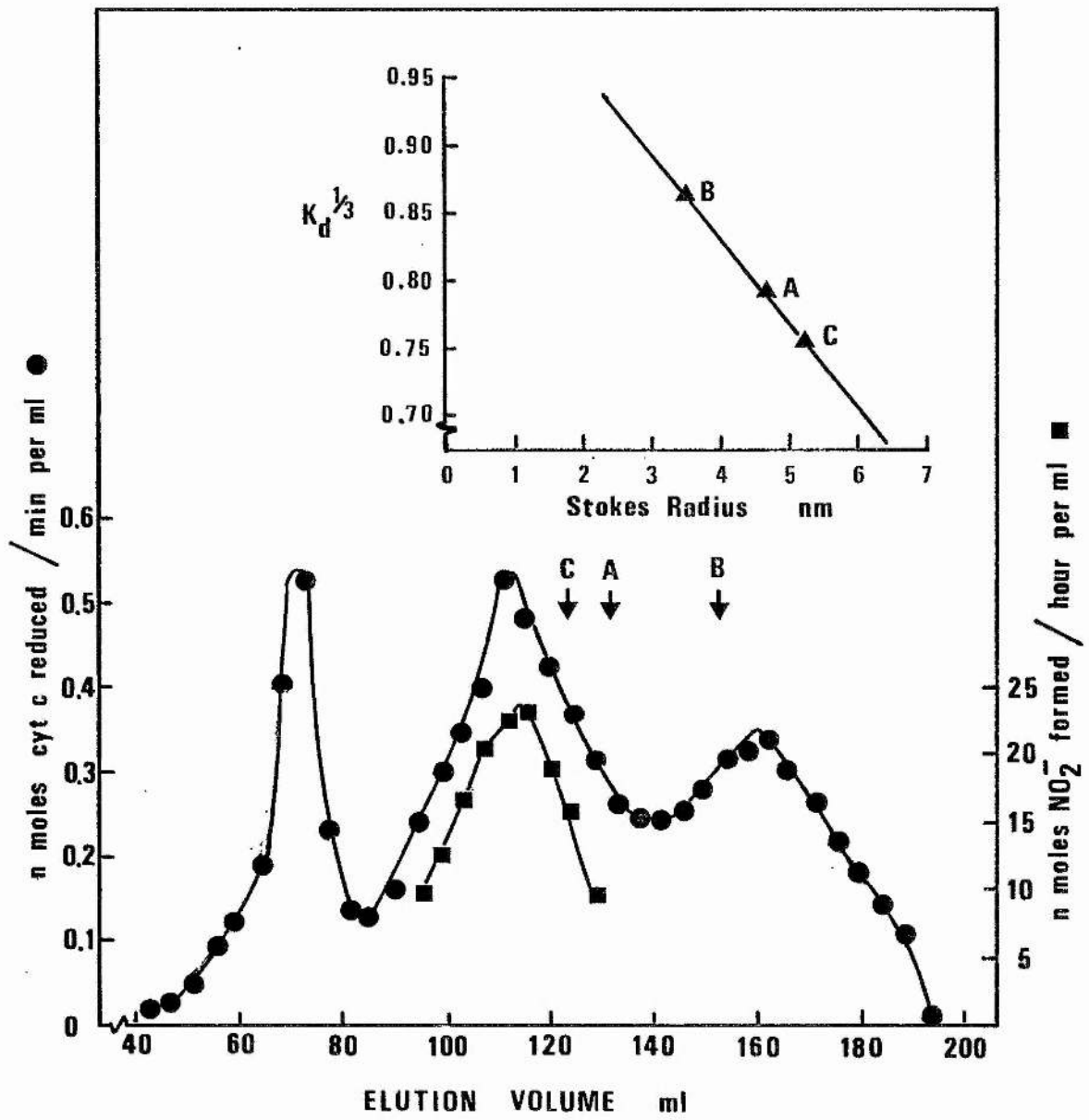
NADH-cytochrome c reductase activity quite different from that obtained from 90-hour old nitrate plants (Fig. 28). There is a marked decrease in the NADH-cytochrome c reductase activity in the 8 S region and an increase in activity in the 3 S region compared to that shown in Fig. 28. The heaviest species (Fig. 34) sediments at 7.7 S and corresponds to intact nitrate reductase, but unlike the result with 90-hour old plants (Fig. 28) there is a shoulder, with a sedimentation coefficient of 6.8 S, on the trailing edge of the 7.7 S peak. The peak of NADH-cytochrome c reductase activity in the 3-4 S region (Fig. 34) is also heterogeneous with apparent peaks of activity at 3.8 S and 3.1 S which are the same values as those observed for the NADH-cytochrome c reductase species which are derived from nitrate reductase (Fig. 32).

Sephadex G200 gel filtration analysis of extracts of 120-hour old nitrate plants (Fig. 35) also resulted in a profile of NADH-cytochrome c reductase activity quite different to that found for 90-hour old nitrate plants (Fig. 29). The first-eluted species again elutes just at the void volume (Fig. 35), however, whereas in Fig. 29 the next-eluted species has a Stokes radius of 6.4 nm, the value observed for 120-hour nitrate plant extracts (Fig. 35) is 5.8 nm. Similarly, the peak of NADH-nitrate reductase activity is shifted from 6.4 nm (Fig. 29) to 5.7 nm (Fig. 35) although there is a shoulder on the leading edge of this 5.7 nm peak which has an approximate Stokes radius of 6.3 nm. The last-eluted peak of NADH-cytochrome c

FIG. 35

Sephadex G200 Gel Filtration of an Extract from 120-hour old Nitrate Plants

This figure shows the distribution of NADH-cytochrome c reductase and NADH-nitrate reductase activities following gel filtration of an extract from 120-hour old nitrate plants through Sephadex G200. C, A and B denote the positions of the reference proteins catalase, alcohol dehydrogenase and bovine serum albumin, respectively.


0.95
0.90
$K_d^{1/3}$ 0.85
0.80
0.75
0.70
B
A
C
0 1 2 3 4 5 6 7
Stokes Radius nm
n moles cyt c reduced / min per ml ●
n moles NO_2^- formed / hour per ml ■
C A B
0 0.1 0.2 0.3 0.4 0.5 0.6
5 10 15 20 25
40 60 80 100 120 140 160 180 200
ELUTION VOLUME ml

reductase activity (Fig. 35) corresponds (with the exception of one point) to a Stokes radius of 3.05 nm which is very close to the value of 3.1 nm found for the major NADH-cytochrome c reductase species to be released from nitrate reductase (Fig. 33).

Thus it is clear that there is a major difference between the types of NADH-cytochrome c reductase species found in extracts from 90-hour old and 120-hour old nitrate plants. The likely reasons for this will be discussed in the General Discussion but it would appear that in extracts from older plants something causes nitrate reductase to be less stable such that the levels of this species decrease while the levels of those NADH-cytochrome c reductase species derived from it increase.

A new species of NADH-cytochrome c reductase is also observed in extracts from older plants, apparently having a sedimentation coefficient of 6.8 S and a Stokes radius of 5.8 nm. That this species can also possess NADH-nitrate reductase activity (Fig. 35) indicates that it, like the 3.9 nm and 3.1 nm species, is also derived from the 7.7 S, 6.4 nm nitrate reductase.

SECTION IV - FURTHER CHARACTERISATION OF THE NADH-CYTOCHROME c REDUCTASE SPECIES DERIVED FROM NITRATE REDUCTASE

The levels of the NADH-cytochrome c reductase species derived from nitrate reductase in extracts from 90-hour old plants (Figs. 32 and 33) were very low, making accurate

determinations of the molecular parameters difficult. In an effort to confirm the sedimentation coefficients provisionally assigned to these species it was decided to make use of the fact that the level of the nitrate reductase-derived species is much elevated in extracts from plants older than 90 hours.

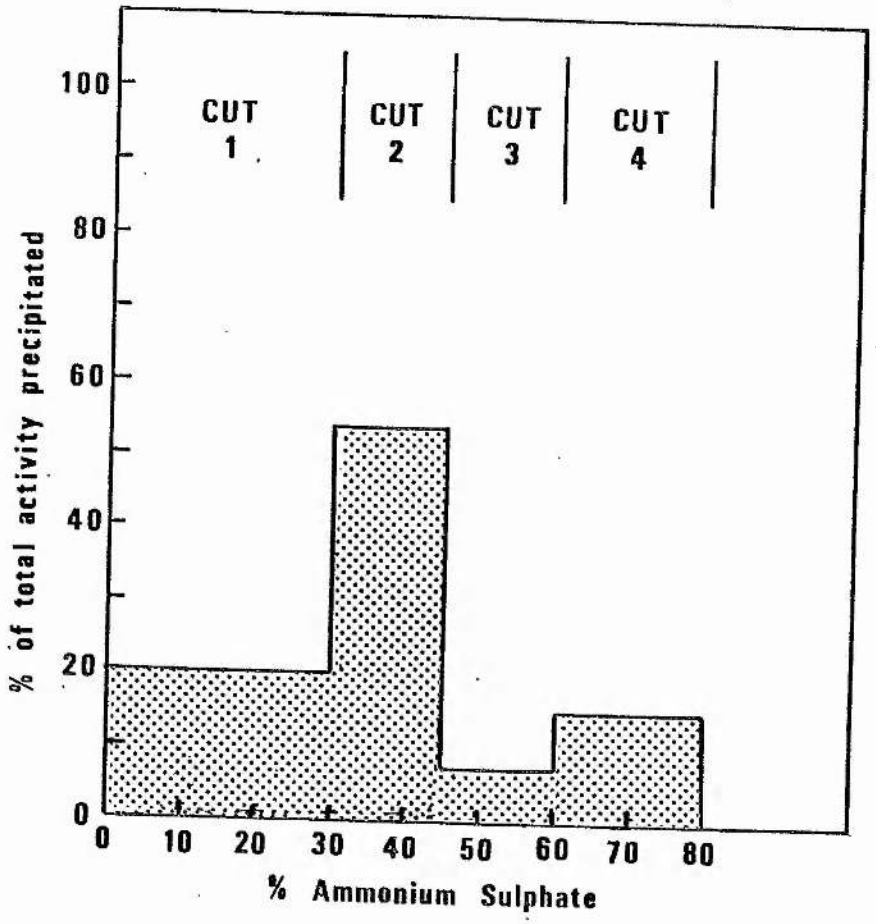
In order to prevent the presence of the constitutive 27 800 molecular weight NADH-cytochrome c reductase from interfering in these determinations, a method was found to remove it from the extract. A series of $(NH_4)_2SO_4$ precipitations from an extract prepared from 90-hour old nitrate plants were assayed for NADH-cytochrome c reductase activity (Fig. 36) and a peak of activity was found to precipitate between 60% and 80% $(NH_4)_2SO_4$ saturation. As the 27 800 molecular weight NADH-cytochrome c reductase species is the smallest NADH-cytochrome c reductase species found in extracts from barley shoots it appeared likely that it would be this species which required the highest concentration of $(NH_4)_2SO_4$ to bring about its precipitation.

This possibility was tested using two 75 g samples of 90-hour old nitrate plants. Following extraction and streptomycin sulphate treatment, a 0-60% $(NH_4)_2SO_4$ precipitate was collected from one sample and a 0-80% $(NH_4)_2SO_4$ precipitate collected from the other. Each precipitate was dissolved in Buffer II to a final volume of 10 ml, passed through a column (4.1 cm × 95 cm) of Biogel A1.5 m and the distributions of NADH-cytochrome c reductase activity

FIG. 36

$(NH_4)_2SO_4$ Precipitation Characteristics of NADH-cytochrome c Reductase Activity

This figure illustrates the required $(NH_4)_2SO_4$ concentrations for precipitation of NADH-cytochrome c reductase activity from barley. 90-hour old nitrate plants were used and the experimental details were exactly as described in the main text for Fig. 16.


CUT 1
CUT 2
CUT 3
CUT 4
100
80
60
40
20
0
% of total activity precipitated
0
10
20
30
40
50
60
70
80
% Ammonium Sulphate

determined (Fig. 37). Whereas for the 0-60% sample no activity was detected after fraction 60, for the 0-80% sample activity was detected up to fraction 65 (Fig. 37). These results are consistent with the conclusion that the 27 800 molecular weight (i.e. the smallest) NADH-cytochrome c reductase species is not precipitated by 60% $(NH_4)_2SO_4$.

In order to further study the NADH-cytochrome c reductase species derived from nitrate reductase, it was first necessary to partially purify them by preparative Biogel A1.5 m gel filtration. This helps to separate the individual species and fractions rich in these can then be further analysed by sucrose density gradient centrifugation.

50 g of 144-hour old nitrate plants were extracted as described in Methods, Section II and, following streptomycin sulphate treatment, the protein precipitating between 0 and 60% saturation with $(NH_4)_2SO_4$ was collected and dissolved in Buffer II. This was then passed through Biogel A1.5 m and the distribution of NADH-cytochrome c reductase activity determined (Fig. 38) and also found to be quite different to that found for 90-hour old plants (Fig. 31).

The first-eluted species (Fig. 38) is the high molecular weight NADH-cytochrome c reductase species and it is followed by a heterogeneous peak the leading edge of which, by comparison with Fig. 34, would be expected to represent intact nitrate reductase whilst the trailing edge probably

FIG. 37

Comparative Biogel Filtration of Barley Extracts

This figure shows the distribution of NADH-cytochrome c reductase activity following Biogel A1.5 cm filtration of both a 0.60% $(NH_4)_2SO_4$ fraction and a 0-80% $(NH_4)_2SO_4$ fraction, obtained from 90-hour old nitrate plants.

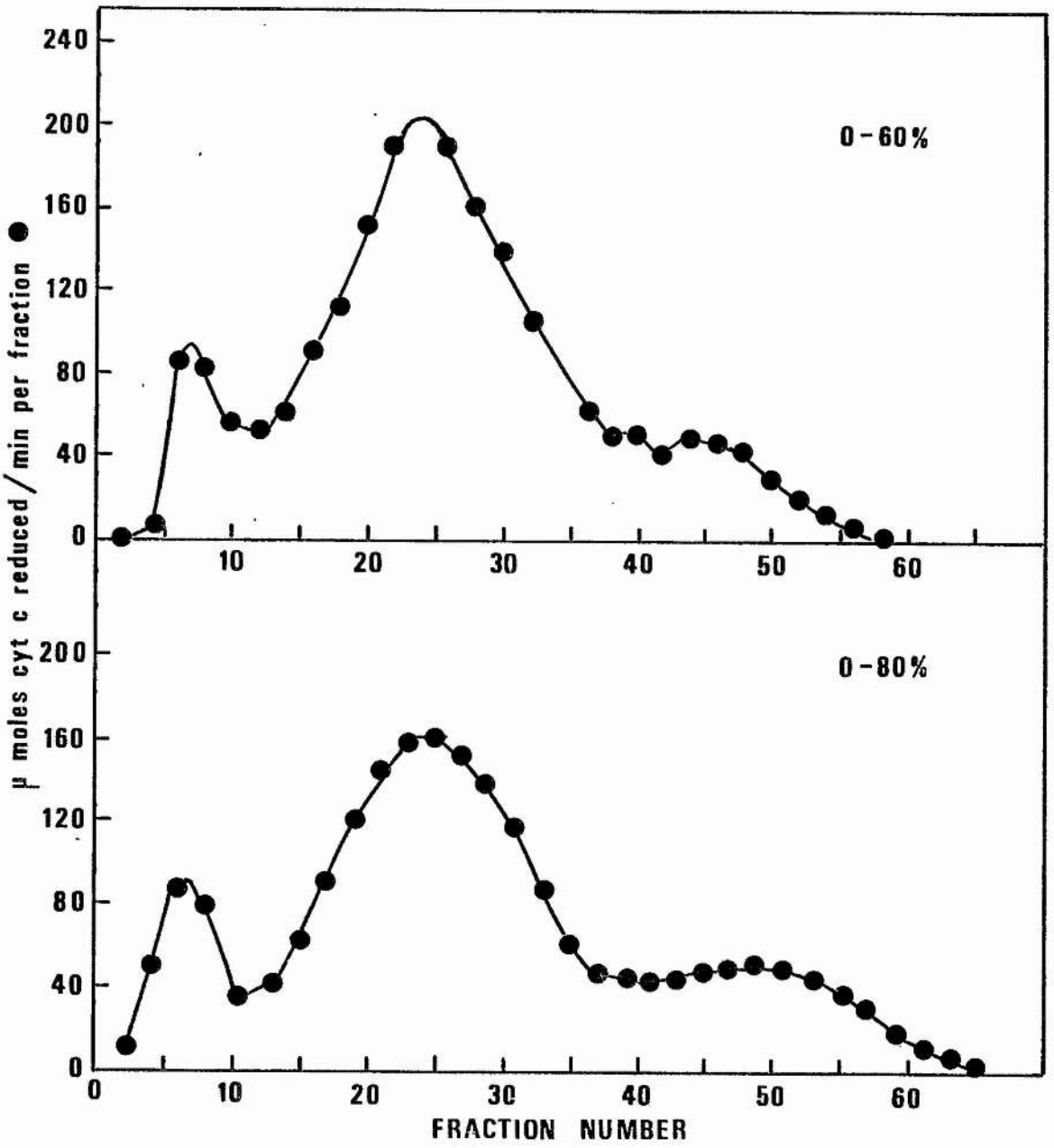
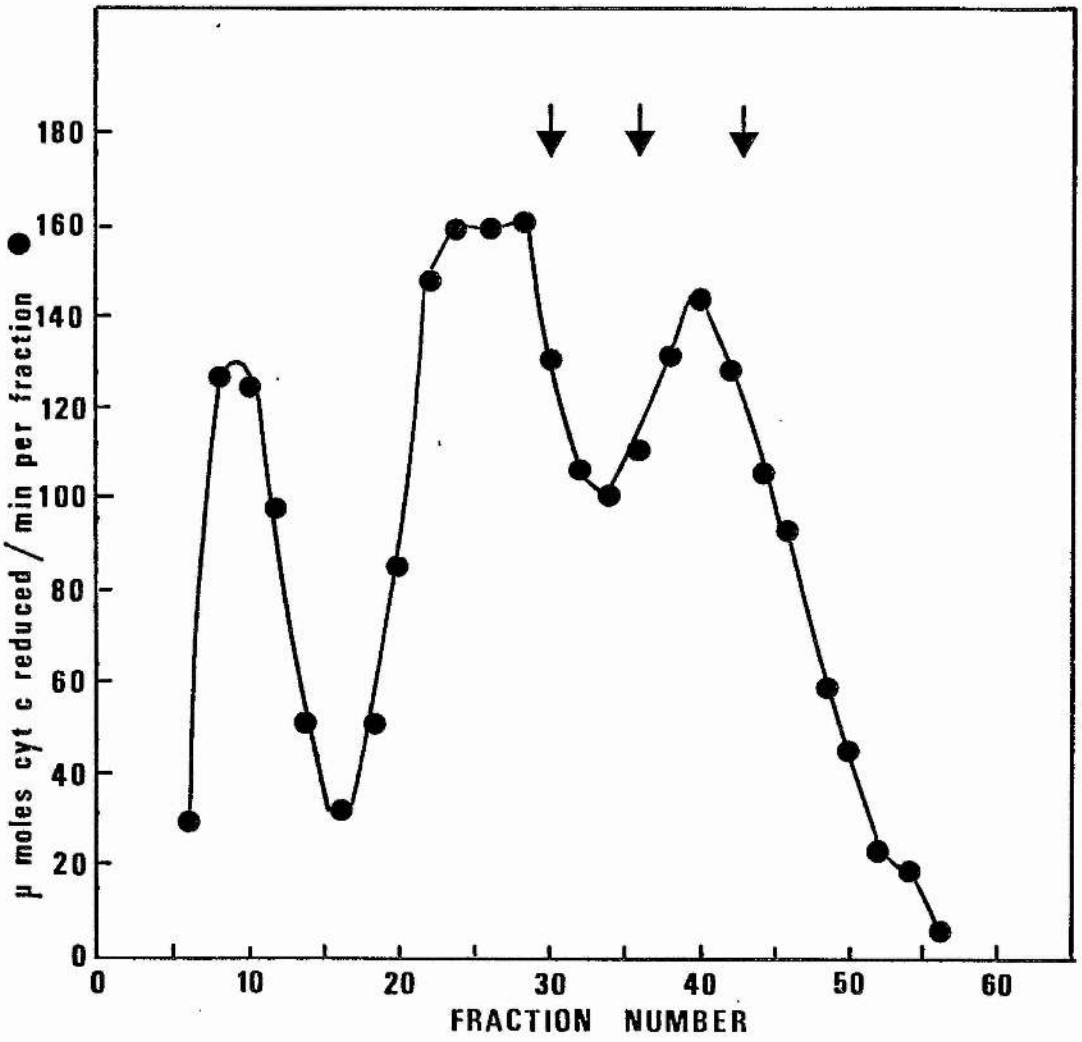

0-60%
0-80%
μ moles cyt c reduced/min per fraction ●
FRACTION NUMBER
0
40
80
120
160
200
240
10
20
30
40
50
60

FIG. 38

Biogel Filtration of an Extract from 144-hour-old Nitrate Plants

This figure shows the distribution of NADH-cytochrome c reductase activity following Biogel A1.5 m gel filtration of an extract from 144-hour old nitrate plants. The arrows denote fractions 30, 36 and 43 which were analysed by sucrose density gradient centrifugation in Figs. 39, 40 and 41 respectively.


180
160
140
120
100
80
60
40
20
0
μ moles cyt c reduced / min per fraction
0
10
20
30
40
50
60
FRACTION NUMBER

represents the 5.8 nm NADH-nitrate reductase/NADH-cytochrome c reductase which is only seen in extracts from plants older than 90 hours. The remaining peak of activity (Fig. 38) represents the smaller (3.9 nm and 3.1 nm) NADH-cytochrome c reductase species derived from nitrate reductase.

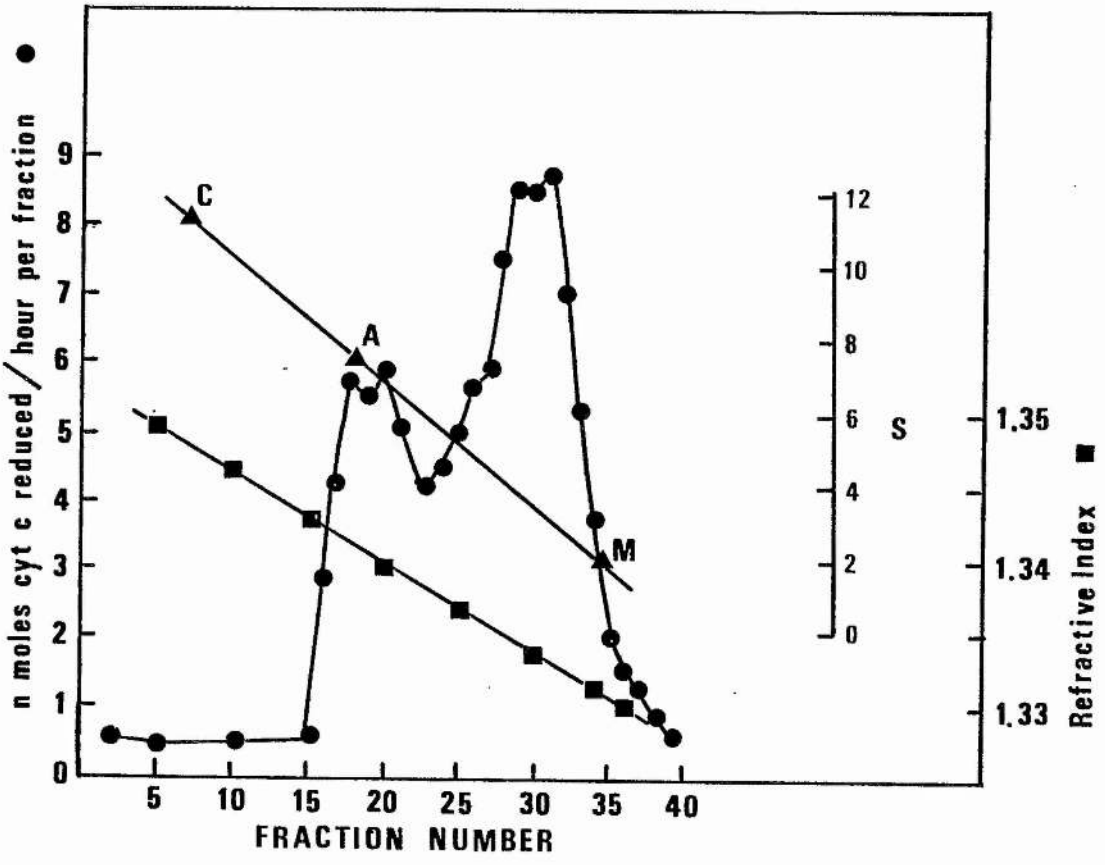
Three fractions from Fig. 38 were analysed: fraction 30 was taken as being rich in the 5.8 nm species; fraction 36 was taken as being rich in the 3.9 nm species, and fraction 43 was taken as being rich in the 3.1 nm species. An aliquot of each of these was subjected to sucrose density gradient centrifugation and the resultant distribution of NADH-cytochrome c reductase determined (Figs. 39, 40 and 41).

Fig. 39 shows the results of analysis of fraction 30 (Fig. 38) and it is immediately obvious that the lighter species of NADH-cytochrome c reductase are present in greater quantities than the heavier species. As the applied sample must have had an excess of the heavier species it may be concluded that at least part of the lighter NADH-cytochrome c reductase species evident in Fig. 39 are derived from the heavier species which were found to have sedimentation coefficients of 7.7 S and 6.8 S. These values are the same as those previously estimated from Fig. 32. The two lighter NADH-cytochrome c reductase species were found to have sedimentation coefficients of 3.8 S and 3.1 S and traces of an additional species were found sedimenting

FIG. 39

Sucrose Density Gradient Centrifugation Analysis of Fraction 30 (Fig. 38)

This figure shows the distribution of NADH-cytochrome c reductase activity following sucrose density gradient centrifugation of an aliquot from fraction 30 (Fig. 38). C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


n moles cyt c reduced / hour per fraction ●
0
1
2
3
4
5
6
7
8
9
C
A
M
5
10
15
20
25
30
35
40
FRACTION NUMBER
12
10
8
6
4
2
0
S
1.35
1.34
1.33
Refractive Index ■

at approximately 5.6 S.

Fig. 40 shows the results of analysis of fraction 36 (Fig. 38). Instead of one major peak of activity being detected corresponding to the species with a Stokes radius of 3.9 nm, as expected, several NADH-cytochrome c reductase species were detected. There is a small peak of activity at 7.7 S, representing what remains of the intact nitrate reductase, and a slightly larger peak of activity at 6.8 S. As in Fig. 39 there is a minor peak of activity at 5.6 S but the major peak has a sedimentation coefficient of 3.1 S. There is, however, a very pronounced shoulder on the leading edge of this peak with a sedimentation coefficient of 3.8 S. It is not, therefore, possible to determine from Fig. 40 whether it is the 3.8 S or the 3.1 S NADH-cytochrome c reductase which has a Stokes radius of 3.9 nm.

Fig. 41 shows the results of analysis of fraction 43 (Fig. 38) and, unlike Figs. 39 and 40 shows the presence of only one species of NADH-cytochrome c reductase, sedimenting at 3.1 S. It is interesting to note that the activity recovered from analysis of fraction 43 (Fig. 41) was much higher (70% yield) than was recovered from analysis of either fractions 30 or 36 (Figs. 39 and 40) (40% yield). This can be interpreted to imply that the 3.1 S NADH-cytochrome c reductase is the most stable of the species examined in this experiment. The presence of significant amounts of this species after sucrose density gradient analysis of fractions 30 and 36, both taken as being rich in

FIG. 40

Sucrose Density Gradient Centrifugation

Analysis of Fraction 36 (Fig. 38)

This figure shows the distribution of NADH-cytochrome c reductase activity following sucrose density gradient centrifugation of an aliquot from fraction 36 (Fig. 38). C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.

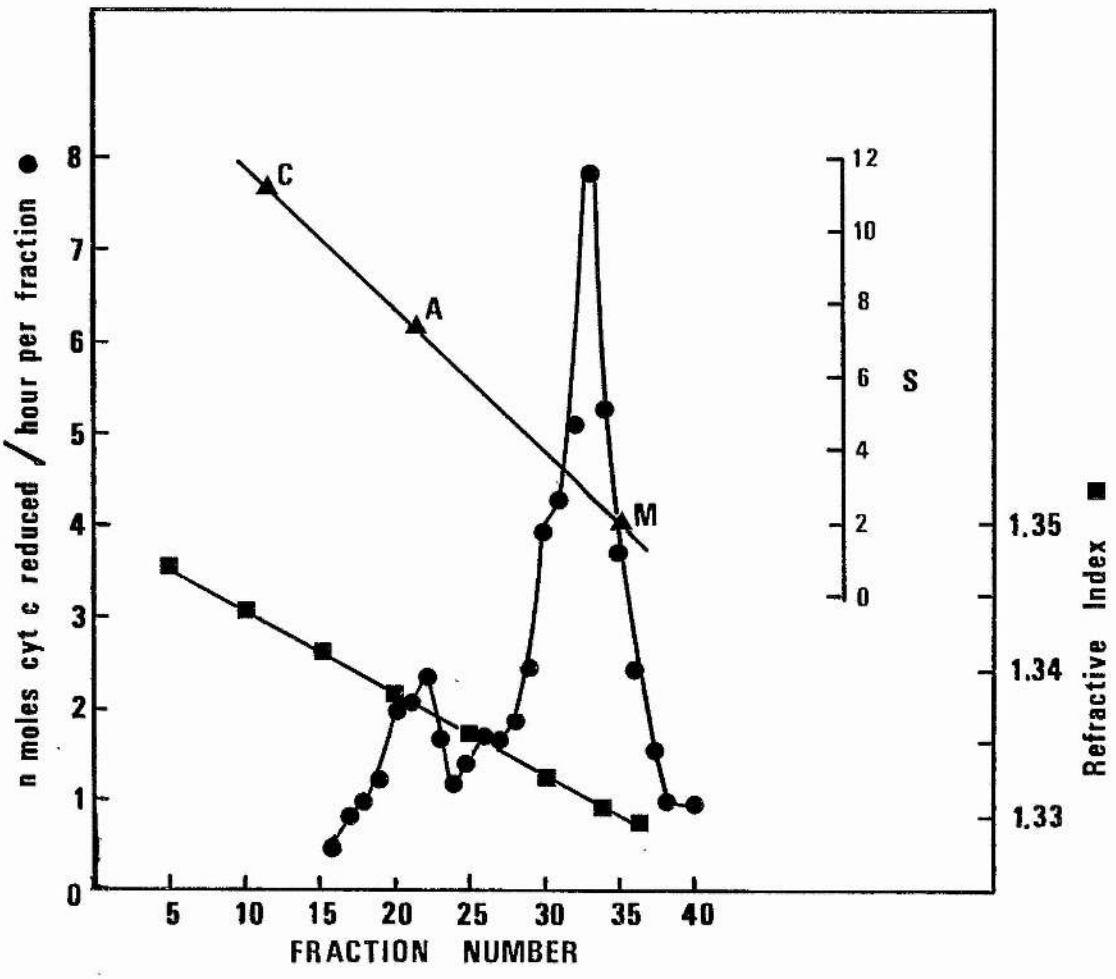
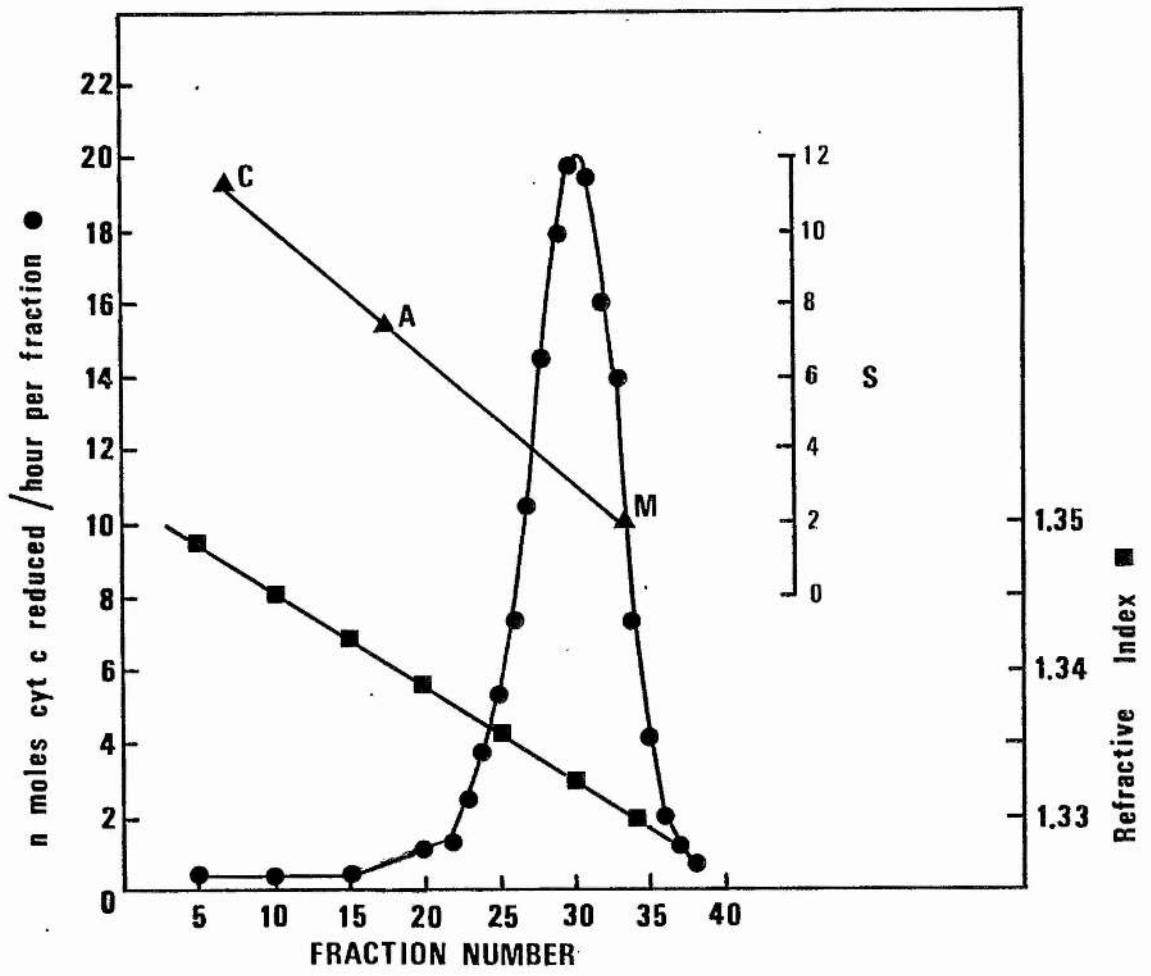

n moles cyt c reduced / hour per fraction ●
0
1
2
3
4
5
6
7
8
C
A
M
12
10
8
6
S
4
2
0
1.35
1.34
1.33
Refractive Index ■
5
10
15
20
25
30
35
40
FRACTION NUMBER

FIG. 41

Sucrose Density Gradient Centrifugation Analysis of Fraction 43 (Fig. 38)

This figure shows the distribution of NADH-cytochrome c reductase activity following sucrose density gradient centrifugation of an aliquot from fraction 43 (Fig. 38). C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


n moles cyt c reduced /hour per fraction ●
22
20
18
16
14
12
10
8
6
4
2
0
C
A
M
12
10
8
6
4
2
0
S
1.35
1.34
1.33
Refractive Index ■
5
10
15
20
25
30
35
40
FRACTION NUMBER

heavier NADH-cytochrome c reductase species, may indicate that the 3.1 S species can be derived from these heavier species. Alternatively, the greater relative stability of the 3.1 S species could equally well account for its detection after sucrose density gradient analysis of fractions 30 and 36 (Fig. 38) if it were present as a contaminant of the applied sample.

SECTION V - MOLECULAR WEIGHTS, FRICTIONAL RATIOS AND AXIAL RATIOS OF NADH-CYTOCHROME c REDUCTASE SPECIES DETECTED IN EXTRACTS FROM NITRATE PLANTS

These molecular parameters were calculated as described in Methods, Section IV. Several distinct NADH-cytochrome c reductase species have been observed in extracts from nitrate plants, the largest of which, with a Stokes radius of 9.1 nm, was also observed in nitrate-less plants (Fig. 3). The sedimentation coefficient of this species was not determined as it is believed to be unrelated to nitrate reductase (Kinsky and McElroy, 1955; Pateman *et al.*, 1964; Sorger, 1966; Wray and Filner, 1970). Without this value the remaining molecular parameters for this species could not be determined.

The major NADH-cytochrome c reductase species found in extracts from nitrate plants was shown to be a partial activity of NADH-nitrate reductase and to have a sedimentation coefficient of 7.7 S, a Stokes radius of 6.4 nm and a calculated molecular weight of 203 000 daltons. This

value is very close to those reported for the nitrate reductases from spinach (Notton, Fido and Hewitt, 1977) and the fungus *Aspergillus nidulans* (Downey, 1971; McDonald and Coddington, 1974). A comparison between the molecular parameters reported for assimilatory nitrate reductases from a variety of sources is given in Table 13.

In order to calculate the molecular weights of the other NADH-cytochrome c reductase species it was necessary to relate the results from gel filtration analysis to those from sucrose density gradient analysis in order that the correct sedimentation coefficient be assigned to each Stokes radius. Figs. 32 and 33 showed that two species of NADH-cytochrome c reductase were derived from nitrate reductase, these having Stokes radii of 3.9 nm and 3.1 nm and sedimentation coefficients of 3.8 S and 3.1 S. It is evident from Figs. 38 and 41 that the 3.1 nm species corresponds to the 3.1 S species, giving a calculated molecular weight of 40 000 daltons. The 3.9 nm species must therefore correspond to the 3.8 S species, giving a calculated molecular weight of 61 000 daltons. The NADH-cytochrome c reductase species, which also possed NADH-nitrate reductase activity, and which was only detected in extracts from plants older than 90 hours was shown (Figs. 34 and 35) to have a sedimentation coefficient of 6.8 S and a Stokes radius of 5.8 nm which correspond to a calculated molecular weight of 163 000 daltons. These values, plus the corresponding frictional ratios and axial ratios for all the NADH-cytochrome c reductase species detected in extracts

TABLE 13

COMPARATIVE PHYSICAL PARAMETERS OF ASSIMILATORY NITRATE REDUCTASES

Organism	Stokes Radius (nm)	Sedimentation Coefficient (S)	Molecular Weight	Frictional Ratio (f/f_o)	Axial Ratio (r_1/r_2)	Reference
Chlorella vulgaris	8.9	9.7	356 000	(1.90)	(16:1)	Solomonson *et al.* (1975)
Chlamydomonas reinhardii	-	10	350 000-500 000	-	-	Sosa, Ortega and Barea (1978)
Neurospora crassa	7.0	8.0	230 000 (231 000)	(1.73)	(13:1)	Garrett and Nason (1969)
	7.0	7.9	228 000	(1.74)	(13:1)	Pan and Nason (1978)
Rhodotorula glutinis	7.05	7.9	230 000	(1.74)	(13:1)	Guerrero and Gutierrez (1978)
Aspergillus nidulans	6.4	7.8	206 000	(1.64)	(11:1)	Downey (1971)
	6.3	7.6	190 000 (197 000)	(1.64)	(11:1)	McDonald and Coddington (1974)
Spinacea oleracea (spinach)	6.0	8.1	197 000 (200 500)	1.55	10:1	Notton, Fido and Hewitt (1977)
Hordeum vulgare (barley)	6.4	7.7	203 000	1.65	11:1	This thesis

Values in parentheses have been calculated as described in Methods (Section IV) from the data presented by the authors.

from barley plants, are shown in Table 14.

It is interesting to note from Table 14 that both nitrate reductase and the three NADH-cytochrome c reductase species derived from it are highly asymmetrical with axial ratios ranging from 11:1 for nitrate reductase down to 6:1 for the 40 000 molecular weight NADH-cytochrome c reductase species. This contrasts with the 27 800 molecular weight NADH-cytochrome c reductase, observed in both nitrate- and nitrate-less plant extracts, which has an axial ratio of only 4:1 and is thus more globular. Thus, asymmetry appears to be a characteristic of the nitrate-reductase associated NADH-cytochrome c reductase species.

Note: The sedimentation coefficient of barley nitrite reductase was found to be 4.2 S (Fig. 42). This compares well with the value of 4.26 S for spinach nitrite reductase determined (Vega and Kamin, 1977) by ultracentrifugation. It can therefore be concluded that none of the NADH-cytochrome c reductase species reported in this chapter are partial activities of nitrite reductase.

DISCUSSION

The original aims of the work reported in this chapter were to confirm the observations of Wray and Filner (1970) and to determine if the 3.7 S NADH-cytochrome c reductase species reported by these authors was either a precursor

TABLE 14

PHYSICAL PARAMETERS OF NADH-CYTOCHROME c REDUCTASE SPECIES DETECTED IN EXTRACTS FROM BARLEY SHOOTS

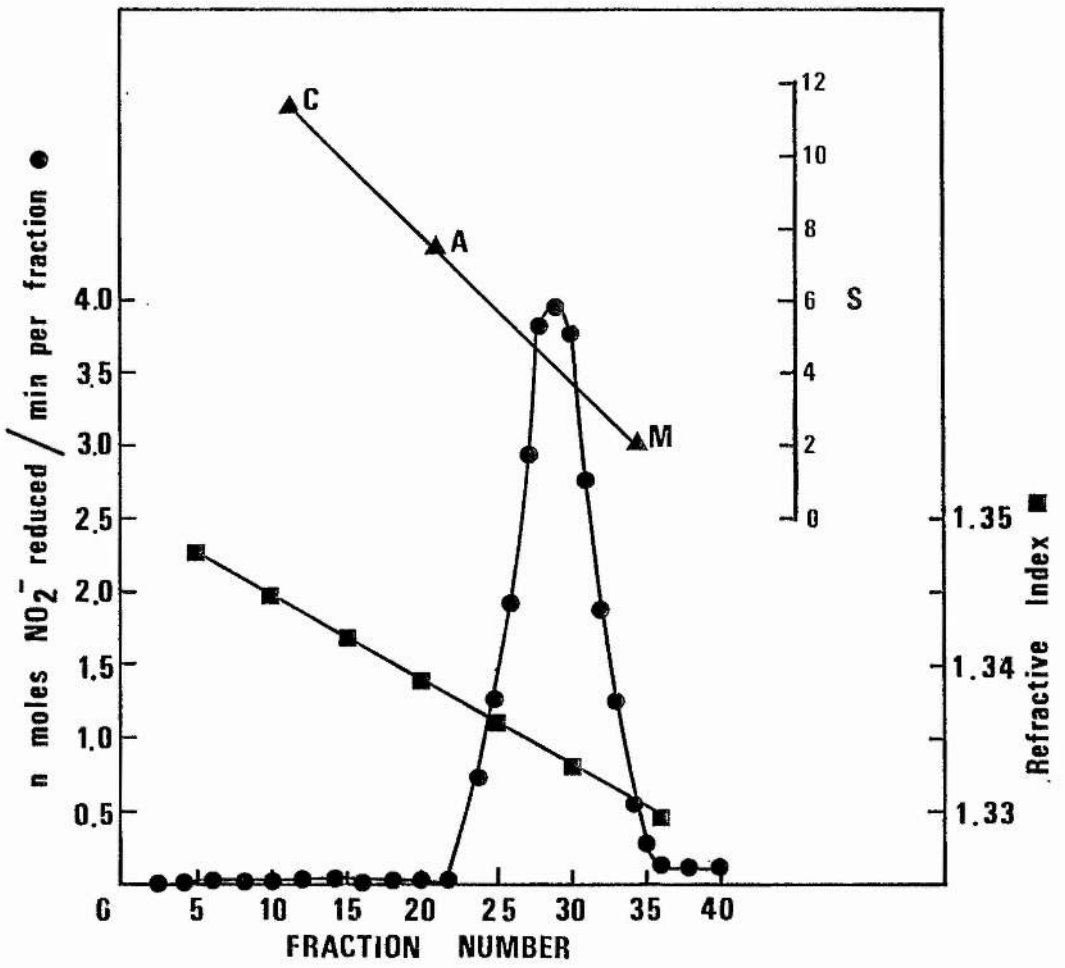
Treatment	Stokes Radius (nm)	Sedimentation Coefficient (S)	Molecular Weight	Frictional Ratio (f/f_0)	Axial Ratio (r_1/r_2)
Nitrate-treated	9.1	ND	-	-	-
	6.4*	7.7	203 000	1.65	11:1
	5.8	6.8	163 000	1.61	10:1
	3.9	3.8	61 000	1.50	8.1
	3.1	3.1	40 000	1.38	6:1
	2.5	ND	-	-	-
Untreated	9.1	ND	-	-	-
	2.5	2.7	27 800	1.25	4:1

ND = not determined * The NADH-nitrate reductase holoenzyme.

FIG. 42

Determination of Sedimentation
Coefficient of Barley Nitrite Reductase

This figure shows the distribution of reduced methyl viologen-nitrite reductase activity following sucrose density gradient centrifugation of an extract from 90-hour old nitrate plants. By reference to the sedimentation of catalase (C), alcohol dehydrogenase (A) and myoglobin (M) the sedimentation coefficient of barley nitrite reductase was determined to be 4.2 S. Sedimentation is from right to left.


C
A
M
12
10
8
6
S
4
2
0
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
5
10
15
20
25
30
35
40
FRACTION NUMBER
n moles NO_2^- reduced / min per fraction ●
1.35
1.34
1.33
Refractive Index ■

subunit of nitrate reductase or a breakdown product of that enzyme, or conceivably both of these. The existence of this species in extracts from rice shoots was confirmed by Shen (1972) but its sedimentation coefficient was not reported.

The sedimentation coefficients reported in this chapter differ from those reported by Wray and Filner (1970) for two major reasons. The first of these is the use here of much larger sucrose gradients which allow a much more accurate determination of the sedimentation coefficient to be made. The second reason is because a different method to that used by Wray and Filner (1970) has been used for the calculation of sedimentation coefficients. Wray and Filner (1970) used the method of Martin and Ames (1961) which relies on comparison with the rate of sedimentation of only one reference protein of known sedimentation coefficient. When the reliability of this method was checked it was found that the results obtained when two standards were included were different for each standard. Thus, by reference to the sedimentation of catalase (the reference protein used by Wray and Filner, 1970) a sedimentation coefficient of 8 S was obtained for barley nitrate reductase, in agreement with the results of Wray and Filner (1970). However, comparison with the sedimentation of alcohol dehydrogenase yielded a value of about 7.8 S for barley nitrate reductase. A similar problem with the method of Martin and Ames (1961) has been reported by

Garrett and Nason (1969) with the nitrate reductase from *N. crassa*.

In an effort to overcome this variability, a method was developed for the determination of sedimentation coefficients based on the sedimentation characteristics of three reference proteins which were included in all gradients. From these, a linear calibration plot for each gradient could be compiled from which accurate and reproducible sedimentation coefficients could be assigned to other proteins in the gradient.

In the work reported here, the sedimentation coefficient found for barley nitrate reductase was 7.7 S (8 S in Wray and Filner, 1970) whilst that of NADH-cytochrome c reductase species C was found to be 3.05 S (3.7 S in Wray and Filner, 1970). The level of activity sedimenting in this region in extracts from nitrate plants was found to be almost double that found in the same region from nitrate-less plants, confirming the results of Wray and Filner (1970).

However, as reported in this Chapter, the sedimentation coefficient associated with this peak differed between extracts from nitrate and nitrate-less plants (Figs. 25 and 28) and this has been explained by the demonstration (Fig. 30) that additional NADH-cytochrome c reductase species are present in extracts from nitrate plants. These new species have been shown (Figs. 32 and 33) to be derived from nitrate reductase. The presence of similar levels of

the 27 800 molecular weight NADH-cytochrome c reductase species in extracts from both nitrate- and nitrate-less plants, together with the demonstration (Fig. 34) that this species cannot be derived from nitrate reductase, argues strongly that this NADH-cytochrome c reductase species is not related to nitrate reductase.

Since the 61 000- and 40 000-molecular weight NADH-cytochrome c reductase species can be derived from nitrate reductase, they undoubtedly represent components of the enzyme. However, these components may be either subunits, associations of subunits or fragments released from the enzyme as a result of proteolytic cleavage. We (Wray, Small and Brown, 1979) have recently presented a model for the structure of higher plant nitrate reductase based on the assumption that these NADH-cytochrome c reductase species represent subunits of the enzyme.

In this model, the 40 000 molecular weight species represents an individual, FAD-containing subunit of nitrate reductase whilst the 61 000 molecular weight species represents an adduct of the 40 000 molecular weight species and cyt b_{557}. This is based on the observation that the cytochrome b from *E. coli* nitrate reductase has an apoprotein molecular weight of 19 500 (MacGregor, 1975). This model postulated the existence of a third, as yet undetected, subunit, with a molecular weight of 40 000, and the structure of nitrate reductase was envisaged to consist of two each of the three types of component. The 160 000

molecular weight NADH cytochrome c reductase species, which also possessed nitrate reductase activity would then represent the intact nitrate reductase lacking either of the 40 000 molecular weight components. The existence of two forms of nitrate reductase from higher plants has been suggested previously following polyacrylamide gel electrophoresis (Ingle, 1968) and isoelectric focusing (Notton, Hewitt and Fielding, 1972) although no evidence for two forms was obtained by Upcroft and Done (1974).

Evidence to suggest that the NADH-cytochrome c reductase species derived from nitrate reductase are more likely to be proteolytic fragments, or domains, than intact subunits of nitrate reductase will be presented in the General Discussion where the consequences of this conclusion will also be examined in relation to the structure of nitrate reductase and other enzymes.

There has only been one report in the literature (Wallace and Johnson, 1978) indicating the possibility that there may be multiple species of NADH-cytochrome c reductase activity sedimenting in the 2-4 S region following sucrose density gradient centrifugation of extracts from nitrate plants. These authors obtained evidence for two species in this region, only one of which was susceptible to attack by a nitrate reductase-specific protease isolated from maize roots (Wallace, 1974, 1975, 1978).

These results strongly suggest that the protease-susceptible NADH-cytochrome c reductase species reported by Wallace and Johnson (1978) is equivalent to the 40 000 molecular weight NADH-cytochrome c reductase species reported here, which has a sedimentation coefficient of 3.1 S and can be derived from nitrate reductase. The other NADH-cytochrome c reductase species reported by Wallace and Johnson (1978) is likely, from its $(NH_4)_2SO_4$ precipitation characteristics, to be equivalent to the 27 800 molecular weight NADH-cytochrome c reductase species reported here.

Evidence supporting these conclusions will be presented in Chapter 4 of these Results, but it is evident that the results of Wallace and Johnson (1978) support the conclusions reached here that there are multiple species of NADH-cytochrome c reductase sedimenting in the 3-4 S region following sucrose density gradient centrifugation of extracts from nitrate plants, and that some of these species may be derived from nitrate reductase.

CHAPTER 3

PURIFICATION OF AN NADH-CYTOCHROME c REDUCTASE SPECIES DERIVED FROM BARLEY NITRATE REDUCTASE

INTRODUCTION

As described in Chapter 2 of these Results, the types of NADH-cytochrome c reductase species observed in extracts from nitrate plants is dependent upon the age of the source tissue. As the plant ages, the amount of extractable nitrate reductase activity decreases and the levels of smaller NADH-cytochrome c reductase species increase. Some of these species have been shown (in Chapter 2) to be derived from nitrate reductase and these conclusions are supported by the observations of Wallace and Johnson (1978) who demonstrated that one of these smaller NADH-cytochrome c reductase species was susceptible to proteolysis by a nitrate reductase-specific protease isolated from maize roots.

However, the results presented in Chapter 2 were derived from plants no older than 144-hours. As will be shown in this chapter, analysis of extracts from 168-hour old nitrate plants shows almost no trace of the heavier nitrate reductase-associated NADH-cytochrome c reductase species and contain large amounts of one of the smaller NADH-cytochrome c reductase species. The results presented in the previous chapter also indicated that the 3.1 S NADH-cytochrome c reductase species was likely to be the most stable of the species derived from nitrate reductase and so it would seem likely that it is this species which is observed in extracts from 168-hour old nitrate plants.

As the 3.1 S NADH-cytochrome c reductase species is likely to represent either a subunit or a functional

domain (see General Discussion) of barley nitrate reductase it was decided to confirm its presence in extracts from 168-hour old nitrate plants and attempt its purification. As the 3.1 S species uses NADH as reductant it is likely that it will bind to Blue Dextran-Sepharose and as the 3.1 S species is derived from nitrate reductase, it may be able to be eluted from Blue Dextran-Sepharose by the same conditions as nitrate reductase (see Chapter 1).

Purification of the 3.1 S NADH-cytochrome c reductase species would allow its full characterisation with respect to possible flavin and cytochrome involvements. The absence of a cytochrome would show that this component was not required for NADH-cytochrome c reductase activity although its involvement in the same activity in intact nitrate reductase could not be ruled out.

RESULTS

SECTION I - IDENTIFICATION OF THE MAJOR NADH-CYTOCHROME c REDUCTASE SPECIES IN EXTRACTS FROM 168-HOUR OLD NITRATE PLANTS

50 g of 168-hour old nitrate-plant shoots were harvested and protein extracted by grinding in a mortar with Buffer I (3 ml buffer/gram shoots). The brei was squeezed through a double layer of muslin, treated with streptomycin sulphate as described in Methods, Section II, and then centrifuged at 40 000 g for 45 minutes. The resulting supernatant was adjusted to 60% saturation with $(NH_4)_2SO_4$ and, after stirring

at 4°C for 30 minutes, precipitated protein (which does not include the 27 800 molecular weight NADH-cytochrome c reductase species) was collected by centrifugation and dissolved in Buffer II to a final volume of 14 ml. This was then applied to a column (4.1 cm × 108 cm) of Biogel A1.5 m previously equilibrated in Buffer II and the distribution of NADH-cytochrome c reductase activity among the fractions determined (Fig. 43).

It is immediately obvious that this profile (Fig. 43) is quite different from that obtained from either 90-hour old (Fig. 31) or 144-hour old (Fig. 38) nitrate plants. The first-eluted species is the same in all three Figures, and is the very high molecular weight, constitutive species which is unlikely to be related to nitrate reductase. The progressive loss of the nitrate reductase- associated species is evident as the plant ages with the result that almost no trace of these are present in the extract from 168-hour plants (Fig. 43). Hence the major species in these extracts appears to be the smallest species present.

This species was identified by subjecting an aliquot of fraction 46 (Fig. 43) to sucrose density gradient centrifugation as described in Methods, Section IV. This resulted (Fig. 44) in an almost homogeneous peak of NADH-cytochrome c reductase activity, sedimenting at 3.1 S, with a slight shoulder on the leading edge indicating the presence of small amounts of a heavier (possibly 3.8 S) species.

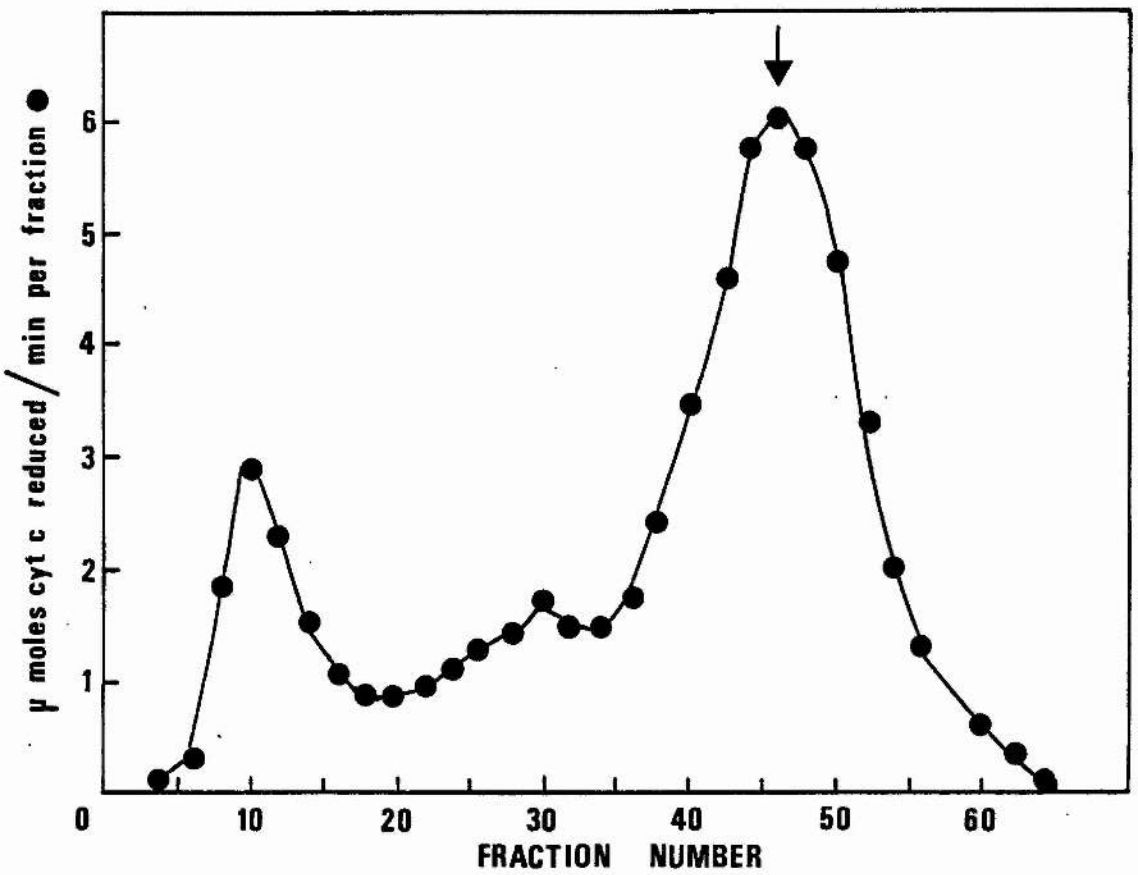

μ moles cyt c reduced / min per fraction ●
0
1
2
3
4
5
6
10
20
30
40
50
60
FRACTION NUMBER

FIG. 43

Biogel Filtration of a 0-60% $(NH_4)_2SO_4$ Fraction Derived from 168-hour old Nitrate Plants

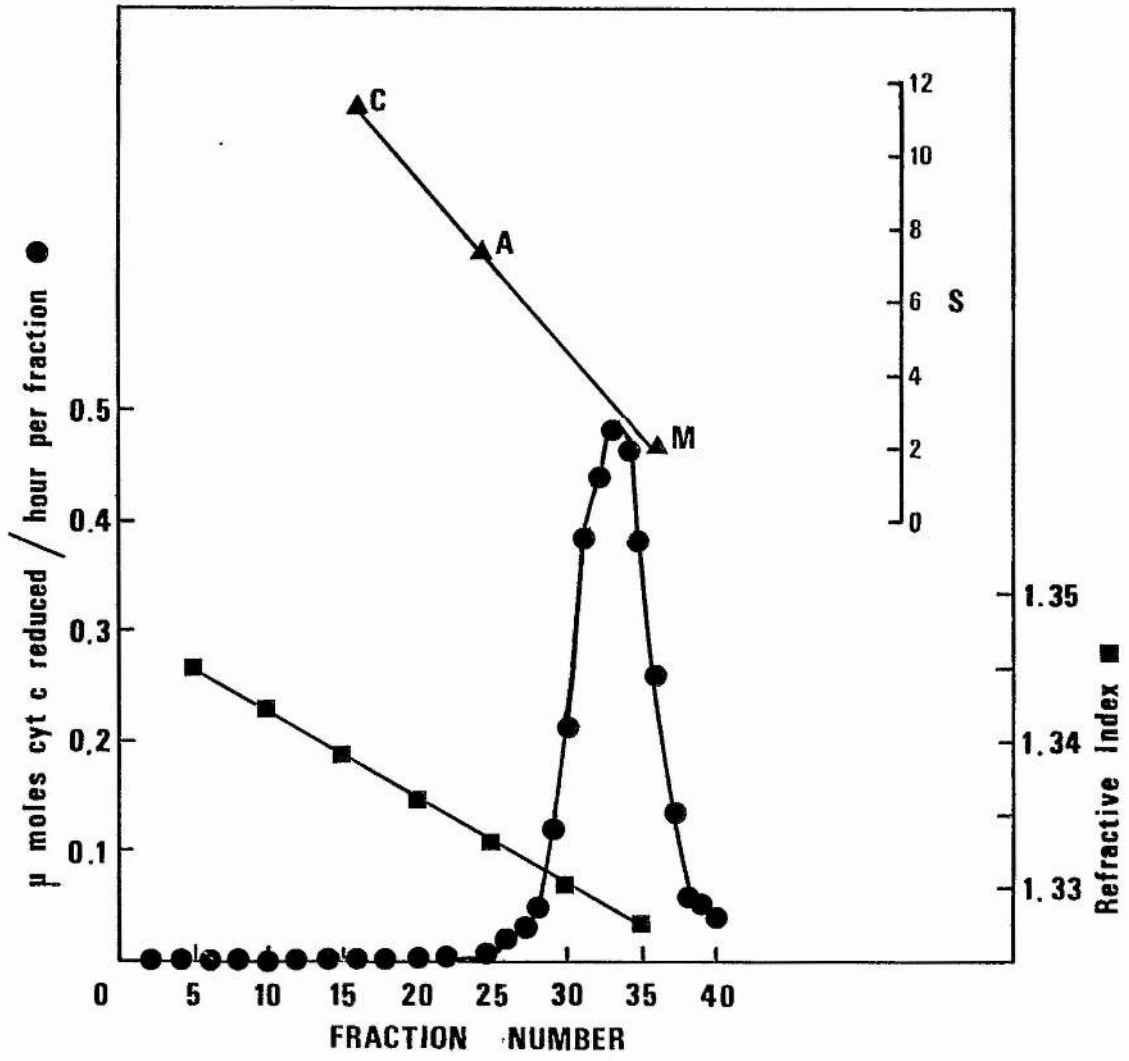
This figure shows the distribution of NADH-cytochrome c reductase activity following Biogel filtration of a 0-60% $(NH_4)_2SO_4$ fraction derived from 168-hour old nitrate plants. The arrow denotes fraction 46 which was analysed by sucrose density gradient centrifugation (Fig. 44).

FIG. 44

Sucrose Density Gradient Centrifugation

Analysis of Fraction 46 (Fig. 43)

This figure shows the distribution of NADH-cytochrome c reductase activity following sucrose density gradient centrifugation of an aliquot from fraction 46 (Fig. 43). C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


C
A
M
12
10
8
6
S
4
2
0
0.5
0.4
0.3
0.2
0.1
μ moles cyt c reduced / hour per fraction ●
1.35
1.34
1.33
Refractive Index ■
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

It is therefore clear that the predominant species of NADH-cytochrome c reductase activity found after analysis of 168-hour old nitrate plants is the 3.1 S species which was shown in Chapter 2 of these Results to have a molecular weight of 40 000 and to be derived from nitrate reductase.

SECTION II - PURIFICATION OF THE 40 000 MOLECULAR WEIGHT NADH-CYTOCHROME c REDUCTASE SPECIES

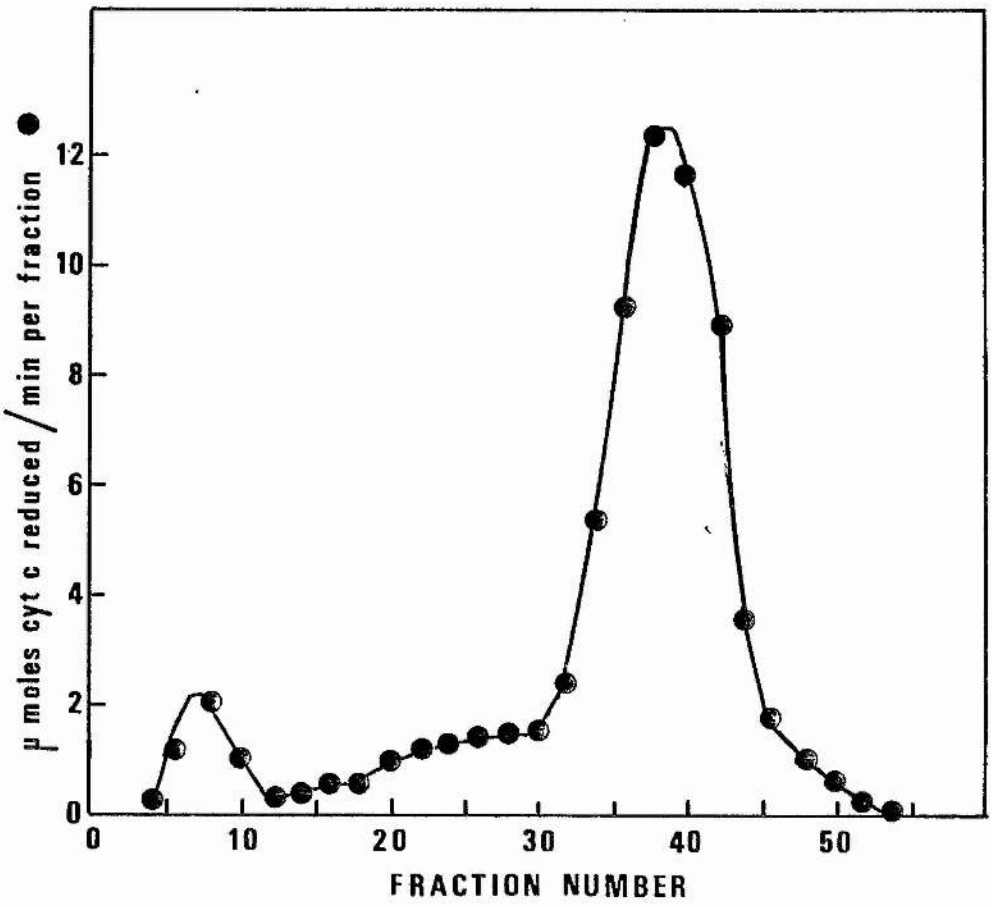
80 g of 168-hour old barley shoots (length 11-12 cm) were ground in a chilled mortar with Buffer II (3 ml buffer/gram shoots) and after squeezing through a double layer of muslin the extract was treated with streptomycin sulphate as previously described and centrifuged at 38 000 g for 50 minutes to remove precipitated material. The resulting supernatant was adjusted to 30% saturation with $(NH_4)_2SO_4$ and precipitated protein, which includes most of the very high molecular weight NADH-cytochrome c reductase species, removed by centrifugation. The resulting supernatant was then adjusted to 60% saturation and precipitated protein again collected by centrifugation. This step removes the 27 800 molecular weight NADH-cytochrome c reductase species.

The precipitated protein was dissolved in Buffer II to a final volume of 20 ml, passed through a column (4.1 cm × 108 cm) of Biogel A1.5 m equilibrated in Buffer II and the distribution of NADH-cytochrome c reductase activity determined (Fig. 45). As expected, only one major species of NADH-cytochrome c reductase activity is present. The

FIG. 45

Biogel Filtration of a 30-60% $(NH_4)_2SO_4$ Fraction Derived from 168-hour old Nitrate Plants

This figure shows the distribution of NADH-cytochrome c reductase activity following Biogel filtration of a 30-60% $(NH_4)_2SO_4$ fraction derived from 168-hour old nitrate plants.


μ moles cyt c reduced / min per fraction
0
2
4
6
8
10
12
0
10
20
30
40
50
FRACTION NUMBER

peak fractions were pooled, the protein precipitated with 60% $(NH_4)_2SO_4$, collected by centrifugation and resuspended in a small volume of Buffer II. Glycerol was then added to a final concentration of 40% (v/v) and the sample then stored at -70°C until further use.

After thawing, the protein was again precipitated with 60% $(NH_4)_2SO_4$, collected by centrifugation, and then dissolved in 20 mM potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA and 10 μM FAD (i.e. Buffer III lacking cysteine). This was then applied to a column (2 cm × 9.5 cm) of Blue Dextran-Sepharose and the column washed until the absorbance of the washings at 280 nm was less than 0.1 following which the column was eluted with 5 μM NADH contained in the same buffer. 6 ml fractions were collected throughout and assayed for NADH-cytochrome c reductase activity (Fig. 46). A large single peak of activity was found, eluting immediately after application of the 5 μM NADH, as was found previously for intact nitrate reductase (Chapter 1 of these Results). The entire peak was pooled and concentrated by dialysis against polypropylene glycol 2025 at 4°C and an aliquot of the concentrated sample removed for protein estimation. Details of the purification achieved are presented in Table 15.

Since there is more than one species of NADH-cytochrome c reductase found in extracts from nitrate plants, the value calculated for the initial specific activity of this purification is rather misleading. Nevertheless, an apparent

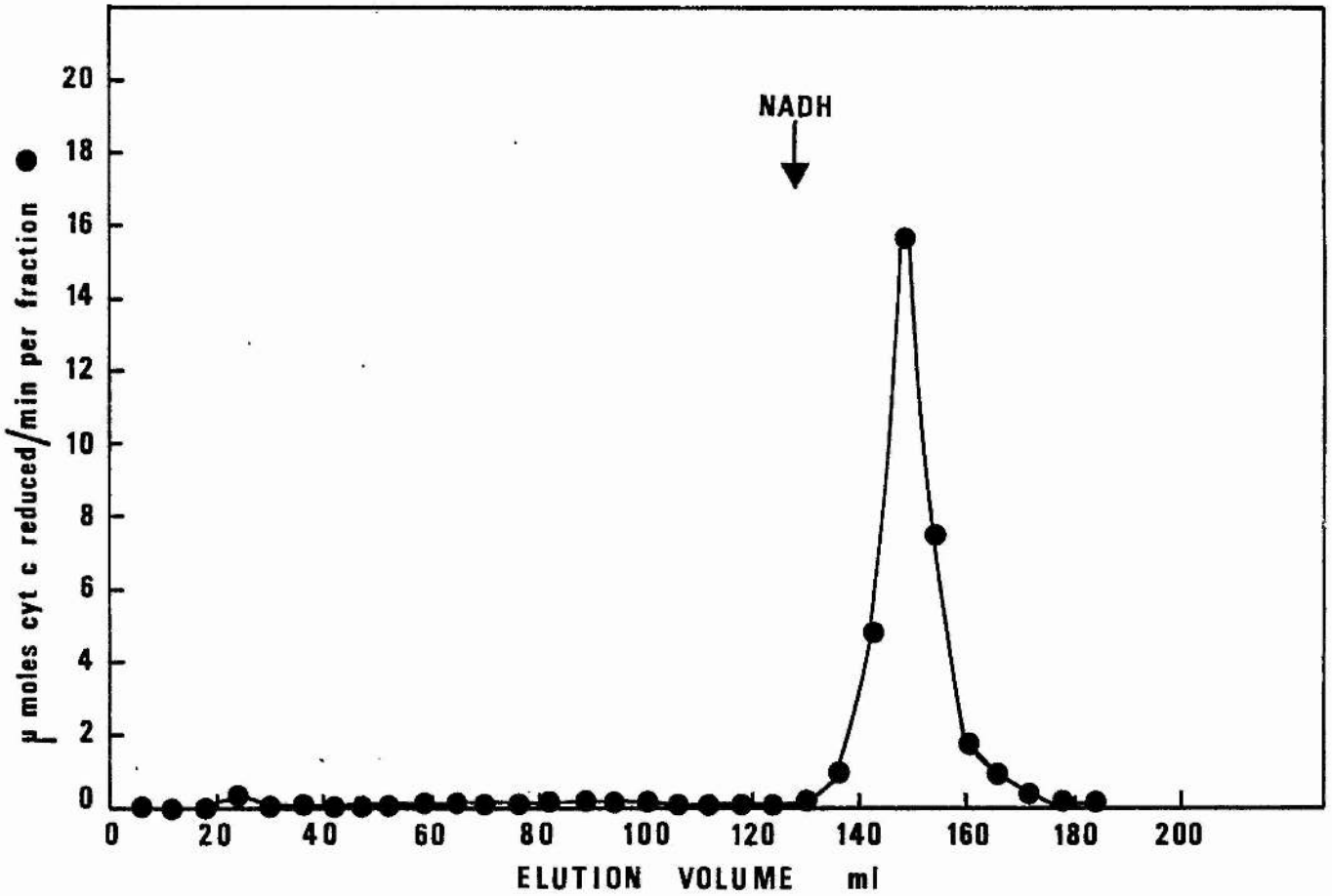

NADH
μ moles cyt c reduced/min per fraction ●
0
2
4
6
8
10
12
14
16
18
20
0
20
40
60
80
100
120
140
160
180
200
ELUTION VOLUME ml

FIG. 46

Blue Dextran-Sepharose Chromatography of Pooled Fractions 33-46 (Fig. 45)

This figure shows the distribution of NADH-cytochrome c reductase following Blue Dextran-Sepharose chromatography of pooled fractions 33-46 (Fig. 45). NADH-cytochrome c reductase activity was eluted with 5 μM NADH contained in Buffer III lacking cysteine. 6 ml fractions were collected from the column (2 cm × 9.5 cm).

TABLE 15

PURIFICATION OF THE 40 000 MOLECULAR WEIGHT NADH-CYTOCHROME c REDUCTASE

Step.	Units*	Protein (mgs)	Specific Activity (units/mg)	Fold Purification	Yield
Extract + Streptomycin sulphate	144.3	1603	0.09	1	100
30-60% $(NH_4)_2SO_4$	102.1	916	0.11	1.24	70.8
Biogel A1.5 m	63.733	287.9	0.238	2.643	43.75
Blue Dextran-Sepharose	29.857	0.6	49.762	552	20.73

*1 unit is defined as 1 µmole cytochrome c reduced per minute

550-fold purification of the 40 000 molecular weight species has been achieved, although it would appear likely that the actual purification is nearer to 1000-fold. Also, as the final specific activity is an average value for the whole peak of activity (Fig. 46) it is possible that the peak specific activity was even higher.

The final specific activity of almost 50 units/mg seems quite high, but as no similar purification has been reported in the literature, the significance of this value is difficult to assess. The amount of protein found associated with the purified enzyme (0.6 mg) seems quite high from only 80 g of starting tissue and so the high specific activity may reflect a very high turnover number for the enzyme rather than a very high degree of purity. Nevertheless, the very high yield of almost 47% from the Blue Dextran-Sepharose column is higher than any obtained for nitrate reductase in Chapter 1 of these Results and indicates that large quantities of barley would not be required for the purification of significant amounts of this enzyme.

SECTION III - ELECTROPHORECTIC ANALYSIS OF THE PURIFIED 40 000 MOLECULAR WEIGHT NADH-CYTOCHROME c REDUCTASE SPECIES

50 µl aliquots of the concentrated enzyme sample were subjected to polyacrylamide gel electrophoresis in 5% acrylamide gels as described in Methods, Section V. However, as was the case with the purified nitrate reductase sample

in Chapter 1 of these Results, no protein-staining bands were detected, making it impossible to assess the purity of the sample.

One major band, with an Rf value of 0.30, (Fig. 47) was found to stain for NADH-dehydrogenase activity. This is very close to the value of 0.29 determined for the second band of NADH-dehydrogenase activity found after electrophoresis of the purified barley nitrate reductase (Fig. 22). Thus it would appear likely that this second band of NADH-dehydrogenase activity (Fig. 22) is due to the presence of the 40 000 molecular weight NADH-cytochrome c reductase species in the sample of purified nitrate reductase. This would not be surprising as both proteins are eluted from Blue Dextran-Sepharose by 5 μM NADH.

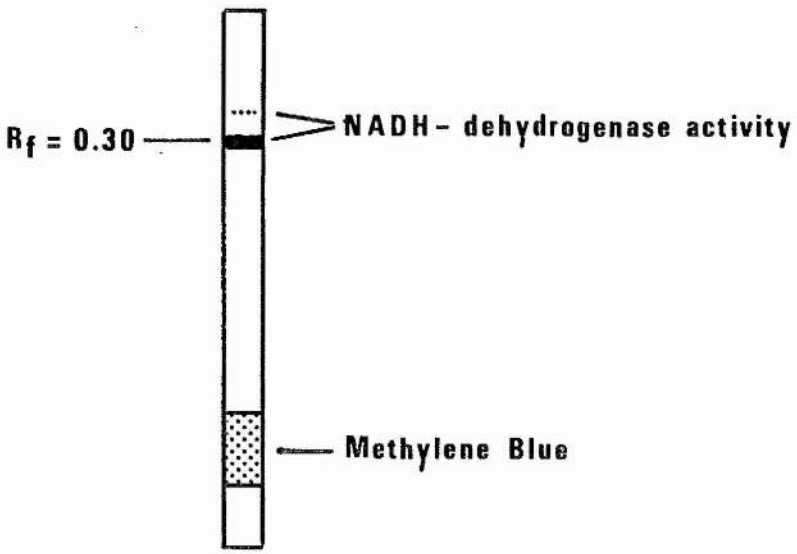
DISCUSSION

As indicated earlier in this thesis, the 40 000 molecular weight NADH-cytochrome c reductase species is likely to be either a subunit or a functional domain of barley nitrate reductase. If this latter explanation is correct then it is likely that the 40 000 molecular weight species is only generated by proteolytic action during extraction of the plant material and the initial purification stages. This fact, together with the existence of other NADH-cytochrome c reductase species in the extract, results in the concept of 'initial specific activity' becoming meaningless. Similarly, the degree of purification

FIG. 47

Electrophoretic Examination of Purified NADH-cytochrome c Reductase

This figure shows a diagrammatic representation of a polyacrylamide gel following electrophoresis of a sample of purified NADH-cytochrome c reductase. The gel was stained (see Methods, Section V) for NADH-dehydrogenase activity and the diagram is based on accurate measurements of the gel.


R_f = 0.30
NADH- dehydrogenase activity
Methylene Blue

is also suspect because it is conceivable that more of the 40 000 molecular weight species was present in the purified sample than was in the original extract. As reported in Chapter 2 of these Results, this species appears to be the most stable of the NADH-cytochrome c reductase species derived from nitrate reductase and so the overall 20% yield of activity in the purification reported here may represent close to 100% yield for the 40 000 molecular weight species. It is not possible to determine if this is the case, but that assumption would yield an overall purification in excess of 2000-fold.

It is clear, therefore, that this 40 000 molecular weight NADH-cytochrome c reductase species can be purified to a high specific activity (Table 15) by a very simple three step technique. It would therefore be possible to purify large amounts of this enzyme, allowing very detailed characterisation to be undertaken which would shed light not only upon this protein but also upon nitrate reductase from which it is derived. It should be possible, by determining the absorption spectrum of this 40 000 molecular weight species, to determine if the cytochrome b_{557} of nitrate reductase (Notton, Fido and Hewitt, 1977) is present in this protein. This would settle the argument in the literature (Hewitt, 1975; Notton, Fido and Hewitt, 1977; Maldonado, Notton and Hewitt, 1978; Fido *et al.*, 1979) as to whether the cytochrome is an essential requirement for the NADH-cytochrome c reductase activity of nitrate reductase.

If the cytochrome is absent, then it cannot be essential for NADH-cytochrome c reductase activity but this would not, however, rule out the possibility that the cytochrome is involved when this activity is expressed by intact nitrate reductase.

With sufficient quantities of the purified 40 000 molecular weight NADH-cytochrome c reductase, it would also be possible to both identify and quantify the flavin involvement in this activity and also determine whether any iron-sulphur centres are present.

It would be very interesting to compare the properties of this 40 000 molecular weight species with those of the NADH-cytochrome c reductase component of the benzoate-1, 2-dioxygenase system from *Pseudomonas arvilla C-1* (Yamaguchi and Fujisawa, 1978). This protein has a molecular weight of 38 000, a Stokes radius of 2.7 nm and a sedimentation coefficient of 3.3 S giving a frictional ratio (f/f_0) of 1.20 (for comparison see Table 14). 1 mole of FAD was present per mole of enzyme. 2.1 moles of iron per mole of enzyme and 1.7 moles of labile sulphide per mole of enzyme suggest that the enzyme contains one iron-sulphur centre of plant ferredoxin type with two iron and two labile sulphur atoms. No cytochrome was present, indicating that for this protein at least, a cytochrome is not an essential requirement for the expression of NADH-cytochrome c reductase activity. Both DCPIP and ferricyanide were capable of replacing cytochrome c in this reaction.

Thus this protein would appear to be very similar to the 40 000 molecular weight NADH-cytochrome c reductase species described in this thesis. Further analysis of the barley enzyme will be required before other similarities or differences can be identified.

CHAPTER 4

CHARACTERISATION OF A FERROCYANIDE-ACTIVATED NADH-CYTOCHROME c REDUCTASE SPECIES IN EXTRACTS FROM BARLEY PLANTS

INTRODUCTION

Wallace and Johnson (1978) have reported the existence, in extracts from several plant species, of a ferrocyanide-activated NADH-cytochrome c reductase species which possesses a sedimentation coefficient of about 4 S. This species was discovered during analysis of the other NADH-cytochrome c reductase species found in plant extracts and ferrocyanide was being used to inhibit cytochrome oxidase activity (Yu and Yu, 1976).

Using differential $(NH_4)_2SO_4$ precipitation and DEAE-cellulose ion-exchange chromatography, Wallace and Johnson (1978) were able to demonstrate the existence of at least three species of NADH-cytochrome c reductase (including the ferrocyanide-activated species) which all sedimented in the 3-4 S region following sucrose density gradient centrifugation. One of the ferrocyanide-insensitive species from this region was also shown to be susceptible to attack by a nitrate reductase-specific protease, as described in Chapter 2 of these Results.

It was decided, therefore, to confirm the existence of the ferrocyanide-activated species in barley and to characterise it with respect to sedimentation coefficient and Stokes radius. In this way it could be determined if the ferrocyanide-activated NADH-cytochrome c reductase activity was carried by any of the NADH-cytochrome c reductase species described in Chapter 2 or was instead

carried by a totally different protein. It was also hoped to be able to determine if the ferrocyanide-activated species could be derived from nitrate reductase.

RESULTS

SECTION I - CHARACTERISATION OF THE FERROCYANIDE ACTIVATED NADH CYTOCHROME c REDUCTASE SPECIES

By Sucrose Density Gradient Centrifugation

The existence of the ferrocyanide-activated species in barley was first confirmed in extracts from 90-hour old nitrate plants subjected to sucrose density gradient centrifugation (Fig. 48) as described in Methods, Section IV. The activity associated with this species is very high, approximately 15-fold greater than that of ferrocyanide-insensitive NADH-cytochrome c reductase in the same region of the gradients. The sedimentation coefficient for the ferrocyanide-activated species was determined (Fig. 48) to be 3.9 S, which is different from all the values previously found for NADH-cytochrome c reductase species in barley extracts (Table 14).

Unlike nitrate reductase, the ferrocyanide-activated NADH-cytochrome c reductase species was also detected after sucrose density gradient centrifugation of extracts from nitrate-less plants (Fig. 49). This can be taken as evidence that the ferrocyanide-activated species is not related to nitrate reductase. The sedimentation coefficient

FIG. 48

Sucrose Density Gradient Centrifugation Analysis of the Ferrocyanide-Activated NADH-cytochrome c Reductase Species in Extracts from Nitrate Plants

This figure shows the distribution of ferrocyanide-activated- and normal-NADH cytochrome c reductase activities following sucrose density gradient centrifugation of an extract from 90-hour old nitrate plants. The activity of the ferrocyanide-activated species has been corrected for the presence of ferrocyanide-insensitive activity. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.

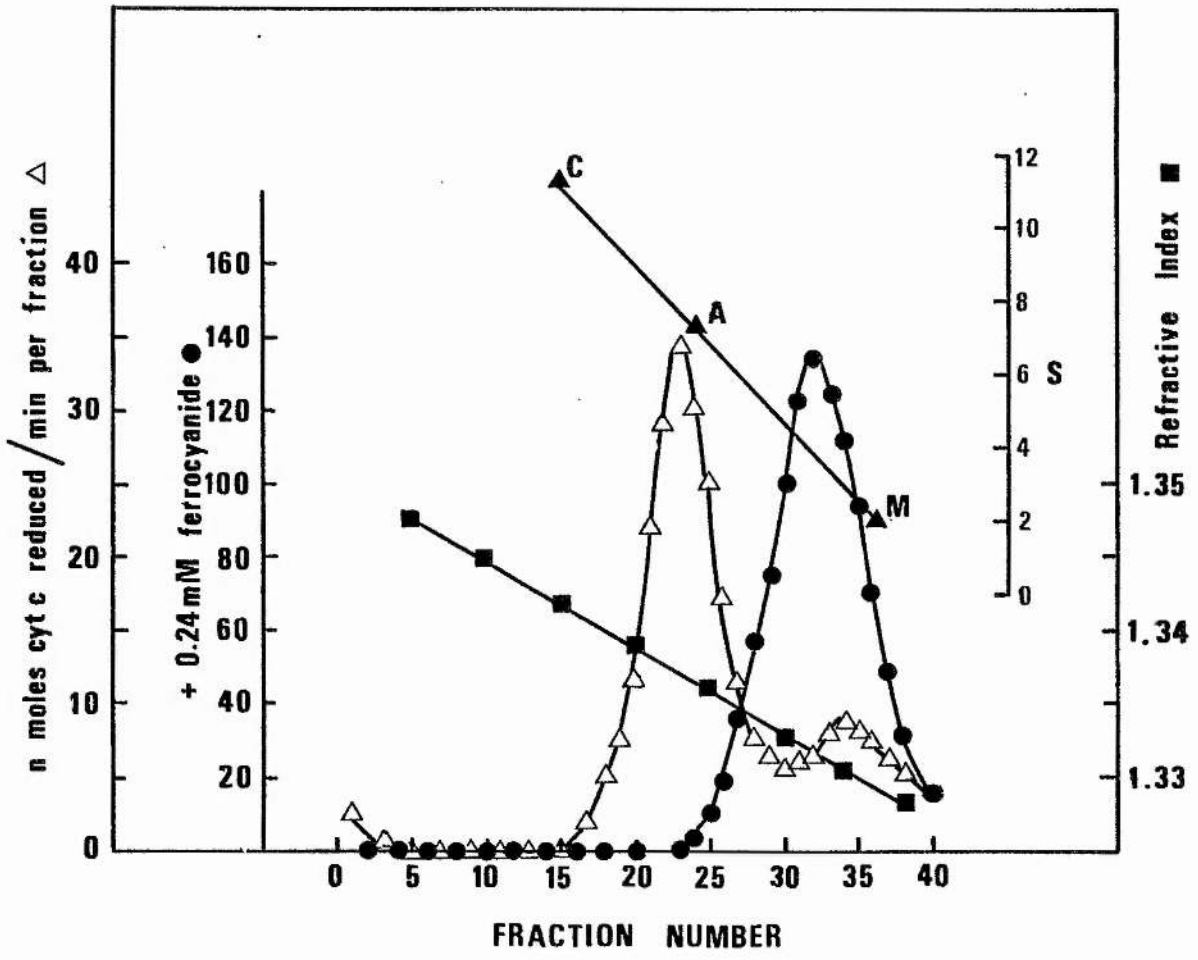
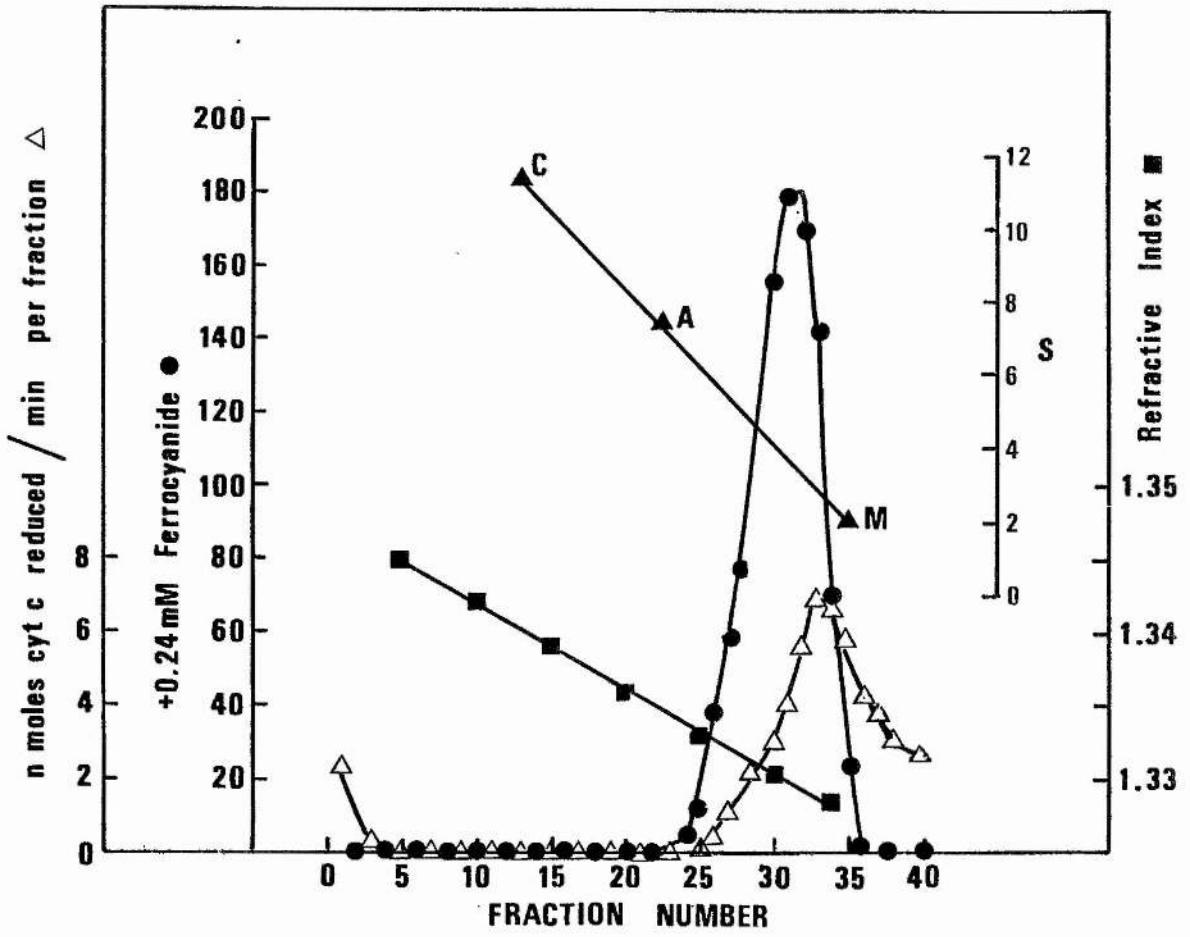

n moles cyt c reduced / min per fraction △
+ 0.24 mM ferrocyanide ●
S
Refractive Index ■
C
A
M
0
5
10
15
20
25
30
35
40
FRACTION NUMBER
1.33
1.34
1.35

FIG. 49

Sucrose Density Gradient Centrifugation Analysis of the Ferrocyanide- Activated NADH-cytochrome c Reductase Species in Extracts from Nitrate-less Plants

This figure shows the distribution of ferrocyanide-activated and normal-NADH cytochrome c reductase activities following sucrose density gradient centrifugation of an extract from 90-hour old nitrate-less plants. The activity of the ferrocyanide-activated species has been corrected for the presence of ferrocyanide-insensitive activity. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


n moles cyt c reduced / min per fraction △
+0.24 mM Ferrocyanide ●
S
Refractive Index ■
C
A
M
FRACTION NUMBER
0
5
10
15
20
25
30
35
40
0
2
4
6
8
20
40
60
80
100
120
140
160
180
200
0
2
4
6
8
10
12
1.33
1.34
1.35

of the ferrocyanide-activated species from nitrate-less plants (Fig. 49) was again 3.9 S, in agreement with the value obtained from nitrate-plants (Fig. 48).

By Sephadex G200 Gel Filtration

An extract from nitrate-less plants was then subjected to Sephadex G200 gel filtration as described in Methods, Section IV. A high level of activity was again obtained, in a single symmetrical peak (Fig. 50) with a Stokes radius of 2.8 nm.

The molecular weight, and other parameters, calculated from the sedimentation coefficient and Stokes radius, of the ferrocyanide-activated NADH-cytochrome c reductase species are presented in Table 16.

SECTION II - CAN THE FERROCYANIDE ACTIVATED NADH-CYTOCHROME c REDUCTASE SPECIES BE DERIVED FROM NITRATE REDUCTASE?

Wallace and Johnson (1978) showed that the ferrocyanide-activated NADH-cytochrome c reductase species was not susceptible to attack by a nitrate reductase-specific protease isolated from maize roots. These authors therefore concluded that the ferrocyanide-activated species was unlikely to be related to nitrate reductase. Further support for this conclusion comes from the observation reported here (Figs. 48 and 49) that the ferrocyanide-activated species is present in extracts from both nitrate-less and nitrate-plants. It was decided, however, to determine if the ferrocyanide-

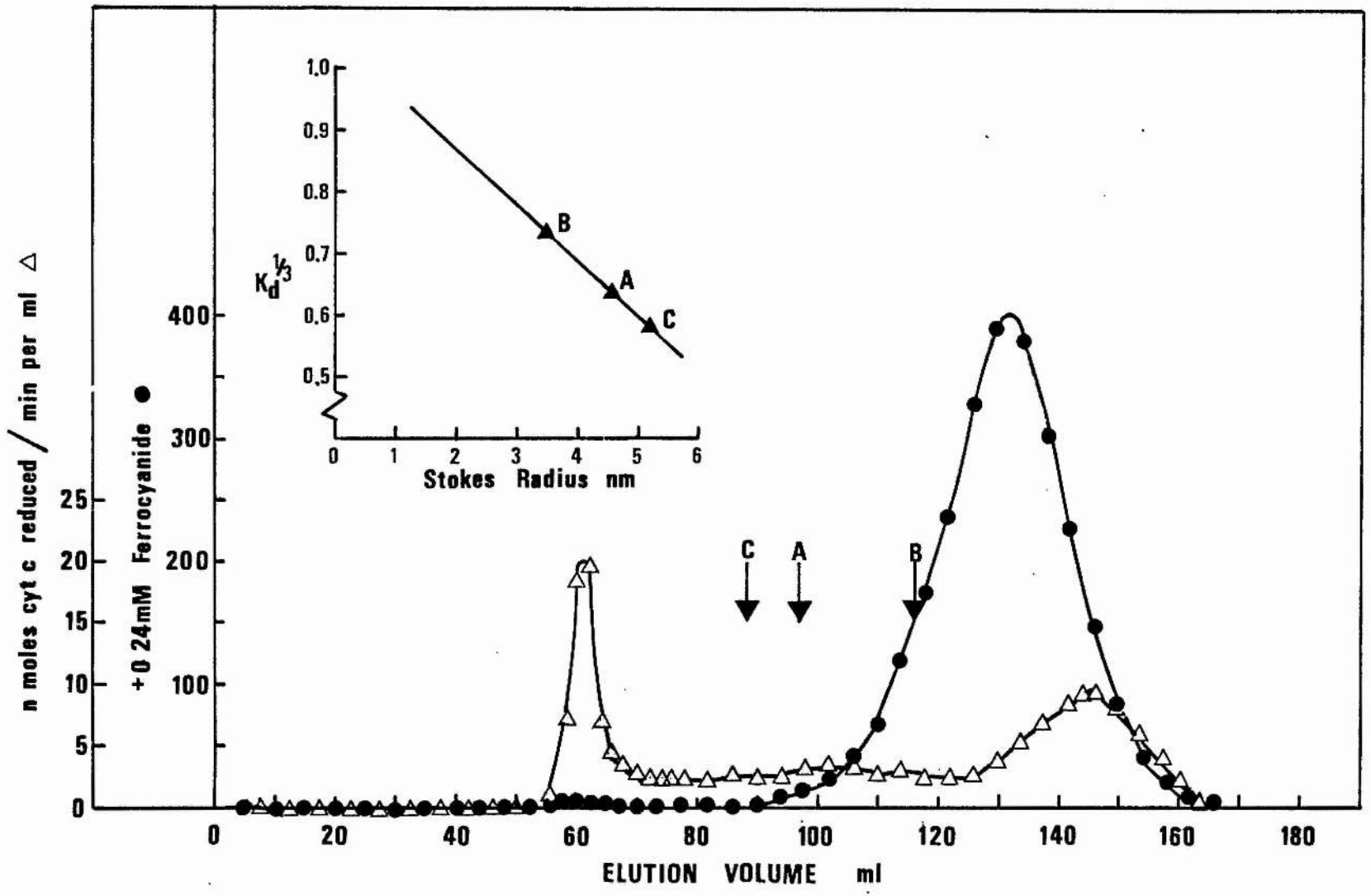

n moles cyt c reduced / min per ml △
+0 24mM Ferrocyanide ●
ELUTION VOLUME ml
Stokes Radius nm
$K_d^{1/3}$
A
B
C
0
20
40
60
80
100
120
140
160
180
0
5
10
15
20
25
100
200
300
400
0.5
0.6
0.7
0.8
0.9
1.0
1
2
3
4
5
6

FIG. 50

Sephadex G200 Gel Filtration Analysis of the Ferrocyanide-Activated NADH-cytochrome c Reductase Species in Extracts from Nitrate-less Plants

This figure shows the distribution of ferrocyanide- activated- and normal-NADH-cytochrome c reductase activities following Sephadex G200 gel filtration of an extract from 90-hour old nitrate-less plants. The activity of the ferrocyanide-activated species has been corrected for the presence of ferrocyanide-insensitive activity. C, A and B denote the positions of the reference proteins catalase, alcohol dehydrogenase and bovine serum albumin, respectively.

TABLE 16

MOLECULAR PARAMETERS OF THE FERROCYANIDE-ACTIVATED NADH CYTOCHROME c REDUCTASE

Sedimentation Coefficient (S)	3.9
Stokes Radius (nm)	2.8
Calculated Molecular Weight	45 000
Frictional Ratio	1.19
Axial Ratio	3.5:1

activated species could be derived from nitrate reductase under conditions where both the 61 000- and 40 000-molecular weight NADH-cytochrome c reductase species are produced (Figs. 31 and 32). An aliquot was therefore taken from the leading edge of the nitrate reductase peak following Biogel filtration (analogous to Fig. 31) and subjected to sucrose density gradient centrifugation as described in Methods, Section IV. The results are presented in Fig. 51 where it can be seen that despite the appearance of both the 3.1 S and 3.8 S NADH-cytochrome c reductase species derived from nitrate reductase, no ferrocyanide-activated activity was detected. Thus it may be concluded that either (a) the ferrocyanide-activated NADH-cytochrome c reductase species is not derived from nitrate reductase and is therefore unlikely to be related to nitrate reductase, or (b) the ferrocyanide-activated species is released from nitrate reductase in an inactive form.

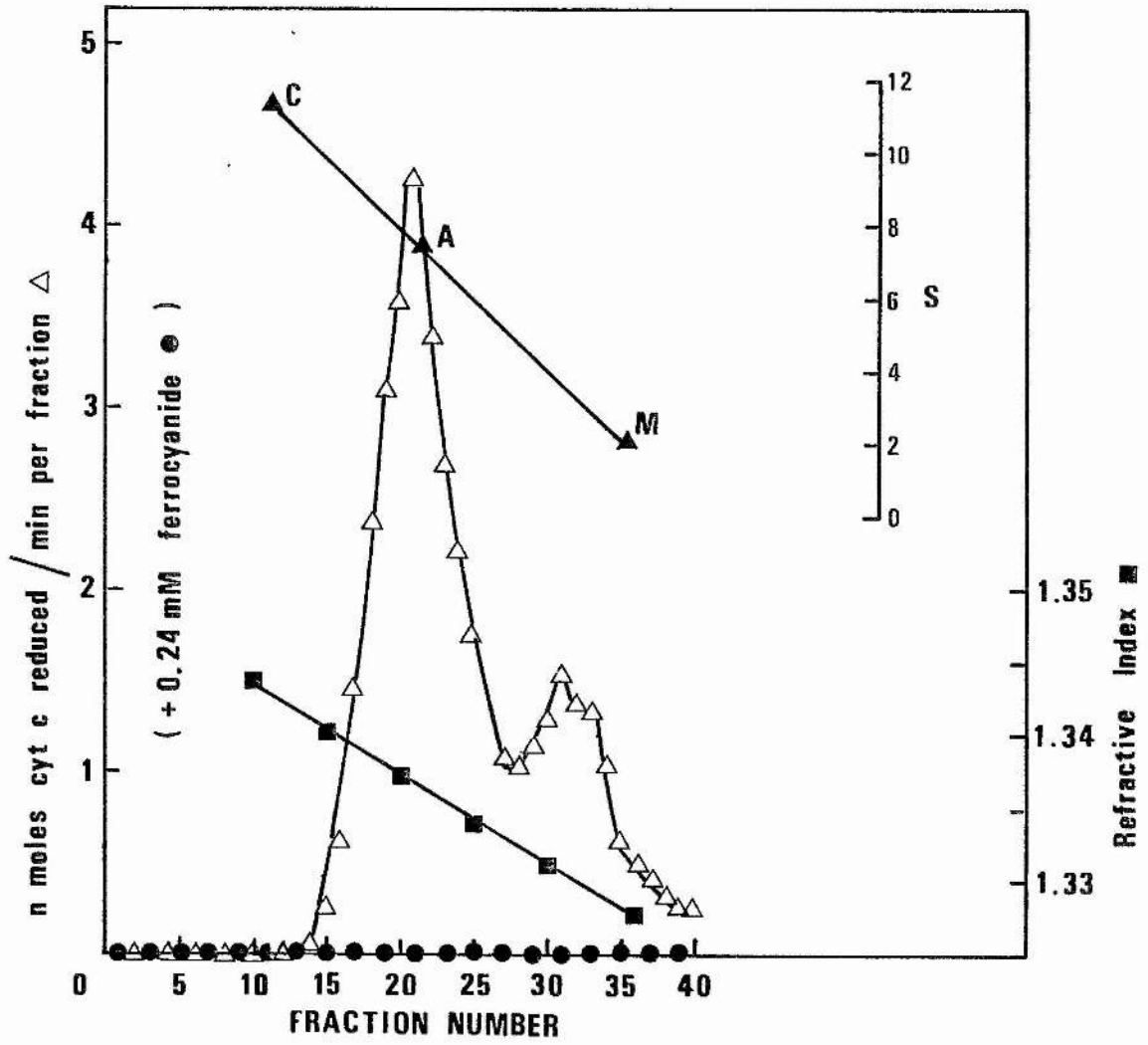
DISCUSSION

The results presented in this section confirm the observation of Wallace and Johnson (1978) that extracts from plant species possess a ferrocyanide-activated NADH-cytochrome c reductase species. As yet, however, no information is available on the role played by this protein *in vivo*, although the available evidence indicates that it is unlikely to be related to nitrate reductase, which itself can catalyse a ferrocyanide-insensitive NADH-cytochrome c reductase activity.

FIG. 51

Sucrose Density Gradient Centrifugation Analysis of NADH-cytochrome c Reductase Species Derived from Nitrate Reductase

This figure shows the distribution of ferrocyanide-activated and normal-NADH cytochrome c reductase activities following sucrose density gradient centrifugation of a aliquot analogous to fraction 16 (Fig. 31). After correction for the presence of ferrocyanide-insensitive activity, no ferrocyanide activated NADH cytochrome c reductase was detected. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


n moles cyt c reduced / min per fraction △
(+ 0.24 mM ferrocyanide ●)
S
Refractive Index ■
C
A
M
FRACTION NUMBER
0
5
10
15
20
25
30
35
40
1
2
3
4
5
0
2
4
6
8
10
12
1.33
1.34
1.35

Wallace and Johnson (1978) used differential $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography to indicate the presence of two ferrocyanide-insensitive NADH-cytochrome c reductase species, in addition to the ferrocyanide-activated species, all sedimenting in the 3-4 S region following sucrose density gradient centrifugation. The $(NH_4)_2SO_4$ precipitation characteristics of both the ferrocyanide-activated and the ferrocyanide-insensitive activities in extracts from 90-hour old barley plants are shown in Fig. 52. It is clear that an $(NH_4)_2SO_4$ concentration in excess of 45% saturation is required to precipitate the ferrocyanide-activated species, whilst the bulk of the ferrocyanide-insensitive activity is precipitated by 45% $(NH_4)_2SO_4$.

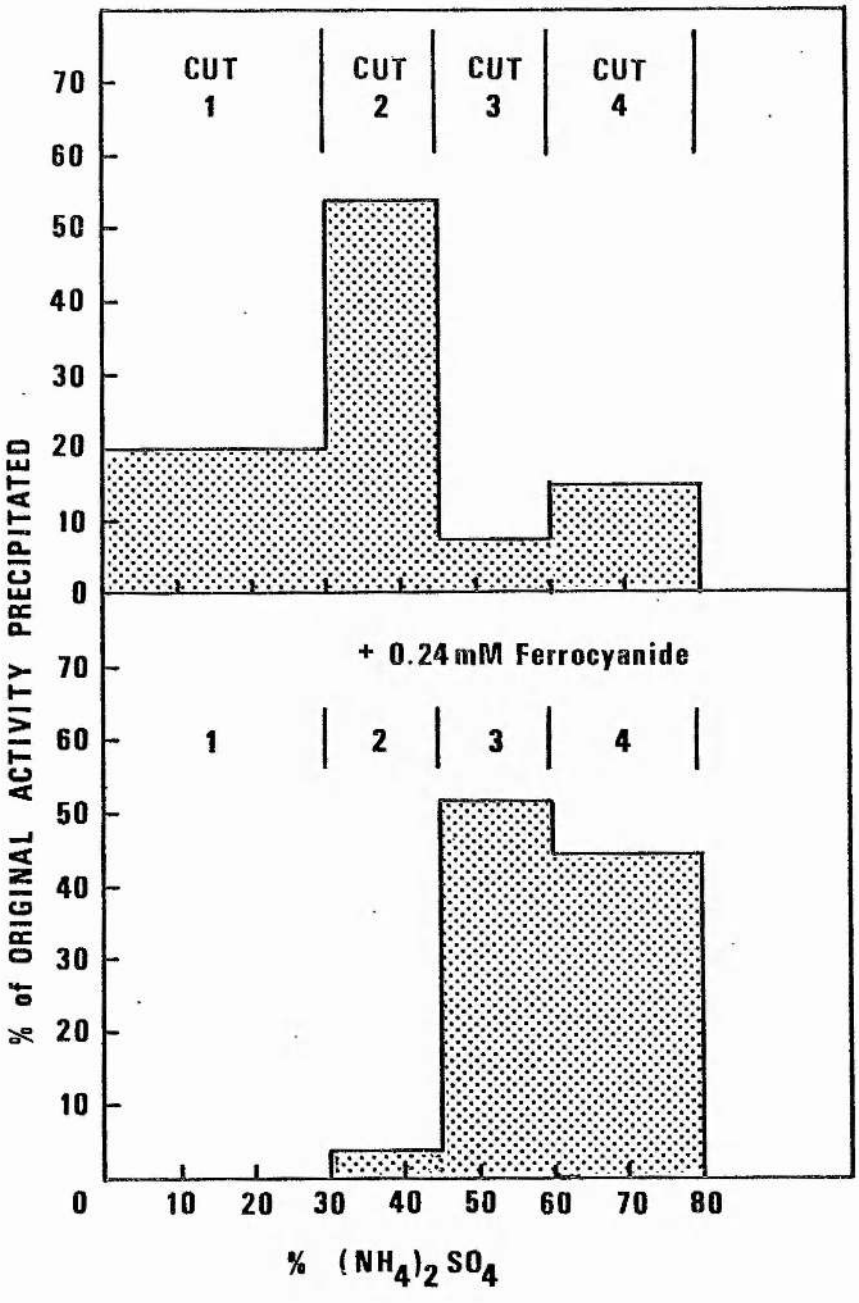
Wallace and Johnson (1978) showed that a precipitate, equivalent to a 45-80% fractionation (Fig. 52) for barley, resulted in the precipitation of both the ferrocyanide-activated species and one ferrocyanide- insensitive species which was not attacked by a nitrate reductase-specific protease isolated from maize roots. As shown in Chapter 2 of these Results, the 27 800 molecular weight NADH-cytochrome c reductase species precipitates between 60% and 80% saturation with $(NH_4)_2SO_4$ and so it would appear likely that it is this species which was detected by Wallace and Johnson (1978).

In a precipitate obtained by the equivalent of 45% saturation with $(NH_4)_2SO_4$ (Fig. 52), the same authors

FIG. 52

$(NH_4)_2SO_4$ Precipitation Characteristics of Ferrocyanide-Activated and Ferrocyanide-Insensitive NADH Cytochrome c Reductase Species

This figure shows the required $(NH_4)_2SO_4$ concentrations for the precipitation of both the ferrocyanide-activated and ferrocyanide-insensitive NADH-cytochrome c reductase activities in extracts from 90-hour old nitrate plants. The experimental details were exactly as described in the main text for Fig. 16.


CUT 1
CUT 2
CUT 3
CUT 4
+ 0.24 mM Ferrocyanide
1
2
3
4
% of ORIGINAL ACTIVITY PRECIPITATED
0
10
20
30
40
50
60
70
80
% $(NH_4)_2 SO_4$

found virtually all the nitrate reductase activity plus one small NADH-cytochrome c reductase species susceptible to attack by a nitrate reductase-specific protease. All the available evidence suggests that this species is likely to be the 40 000 molecular weight species reported in this thesis.

Thus, the results of Wallace and Johnson (1978) have been confirmed and can be explained by the presence of several species of NADH-cytochrome c reductase in extracts from barley plants, as described in Chapter 2 of these Results.

CHAPTER 5

STUDIES ON THE SPECIFICITY OF SOME NADH-DEHYDROGENASE ELECTRON ACCEPTORS FOR BARLEY NITRATE REDUCTASE

INTRODUCTION

As indicated in the Introduction to this thesis, several electron acceptors have been reported to be capable of accepting electrons from the nitrate reductases. These include 2,6-dichlorophenolindophenol (DCPIP) (Kinsky and McElroy, 1955; Garrett and Nason, 1969), ferricyanide (Cove and Pateman, 1967; Solomonson and Vennesland, 1972) and some tetrazolium salts (Cove and Pateman, 1967). However, these results have all been obtained with nitrate reductase from either fungal or algal sources, and not from higher plants. It has generally become accepted, however, by analogy to fungal and algal enzymes, that higher plant nitrate reductase can also catalyse the reduction of these substrates. The evidence in support of this is rather weak.

The ability of higher plant nitrate reductase to reduce DCPIP is generally attributed to Oji and Izawa (1969) who worked with broad bean leaves. Analysis of the data presented by these authors shows that this conclusion is based solely on the fact that a 10-fold purified sample of nitrate reductase also possessed NADH-DCPIP reductase activity. Both activities were almost completely inhibited by 10^{-4} M pCMB and this has been taken as indicating co-identity of the two species.

The ability of higher plant nitrate reductase to reduce tetrazolium salts is attributed (Hewitt, 1975; Notton, Fido

and Hewitt, 1977) to the work of Wang and Raper (1970) who reported on the use of nitroblue tetrazolium (NBT) for the detection of NADH-dehydrogenase isoenzymes from fungal extracts following polyacrylamide gel electrophoresis. However, nitrate reductase was not included in these studies, probably because fungal nitrate reductase uses NADPH rather than NADH as electron donor. Hewitt and co-workers have obviously assumed that because higher plant nitrate reductase possesses an NADH-dehydrogenase activity then it will be capable of donating electrons to NBT.

I could find no evidence in the literature to suggest that ferricyanide can be reduced by higher plant nitrate reductase, other than the observation (Mahler and Cordes, 1971) that ferricyanide is capable of accepting electrons from many dehydrogenases.

Other than nitrate, the only electron acceptor which has been shown conclusively to be capable of being reduced by nitrate reductases from higher plant, algal and fungal sources is cytochrome c (for references, see the Introduction to this thesis).

It was therefore decided to test the ability of some of these dehydrogenase electron acceptors to accept electrons from barley nitrate reductase and to determine their effectiveness relative to cytochrome c.

RESULTS

SECTION I - CORRELATION OF THE DISTRIBUTION OF NADH-DEHYDROGENASE ACTIVITIES WITH THAT OF NITRATE REDUCTASE ACTIVITY FOLLOWING SUCROSE DENSITY GRADIENT ANALYSIS OF UNPURIFIED EXTRACTS FROM BARLEY SHOOTS

Sucrose density gradient centrifugation of crude nitrate-plant extracts was undertaken to determine whether peaks of activity with the various electron acceptors were found to correspond with the peak of nitrate reductase activity. This has been shown previously only with cytochrome c as substrate (Wray and Filner, 1970; Chapter 2 of these Results). The distributions of NADH-DCPIP reductase activity and NADH-cytochrome c reductase activity are presented in Fig. 53 and those of NADH-nitrate reductase and NADH-NBT reductase activities are presented in Fig. 54.

As shown in Chapter 2, the major NADH-cytochrome c reductase species was found to sediment at 7.7 S, as was NADH-nitrate reductase activity. However, it is immediately obvious from both Fig. 53 and Fig. 54 that no peak of dehydrogenase activity with either DCPIP or NBT as substrate is found to sediment at 7.7 S. Rather, the major peak of activity with DCPIP as substrate (Fig. 53) was found to have a sedimentation coefficient of 5.3 S, but it is possible that the high activity associated with this species could mask the existence of additional, less-reactive species. With NBT as substrate (Fig. 54), three

FIG. 53

Distribution of NADH-cytochrome c Reductase and NADH-DCPIP Reductase Activities in Extracts from 90-hour old Nitrate Plants

This figure shows the distribution of NADH-cytochrome c reductase and NADH-DCPIP reductase activities following sucrose density gradient centrifugation of an extract from 90-hour old nitrate plants. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.

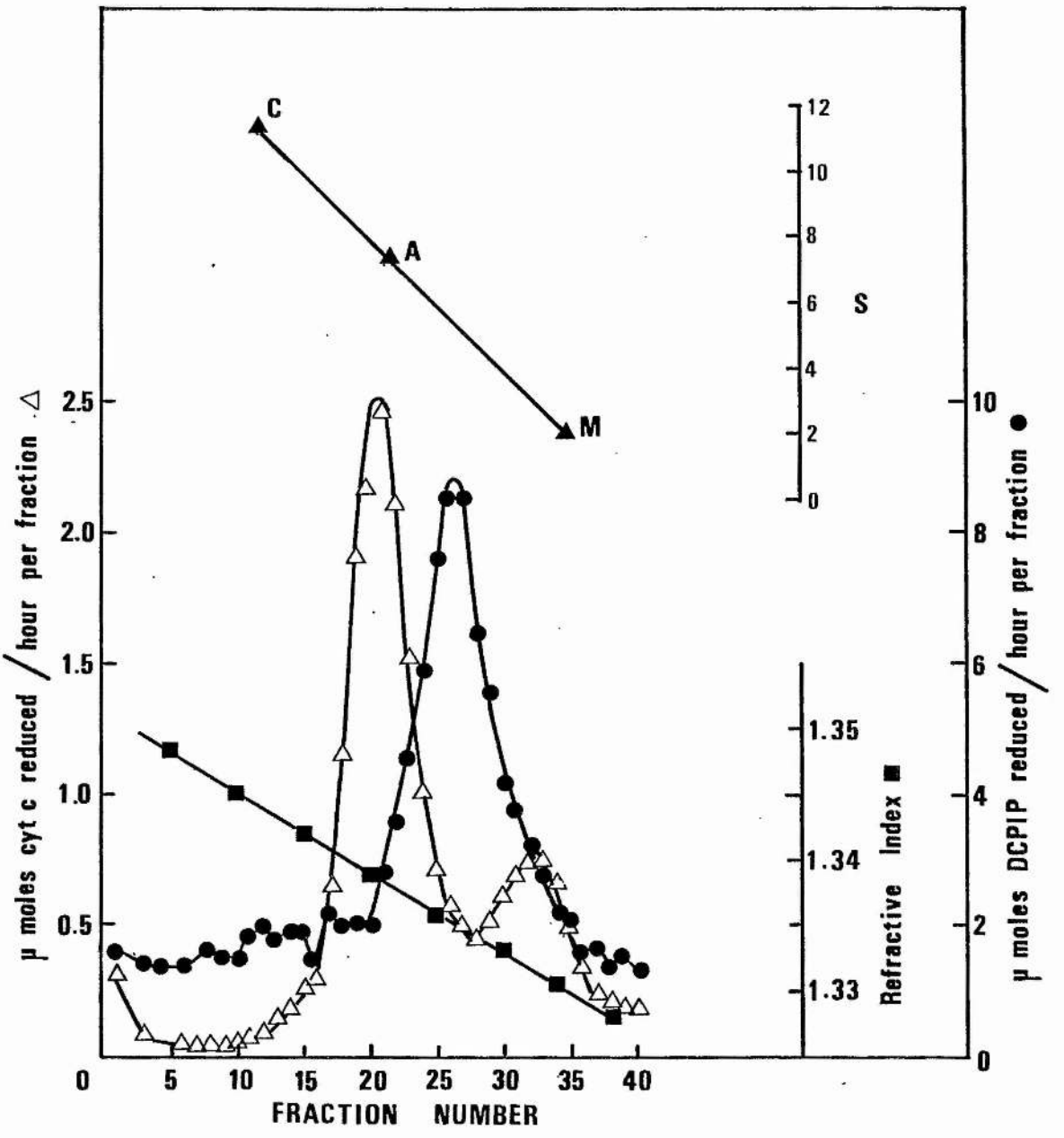
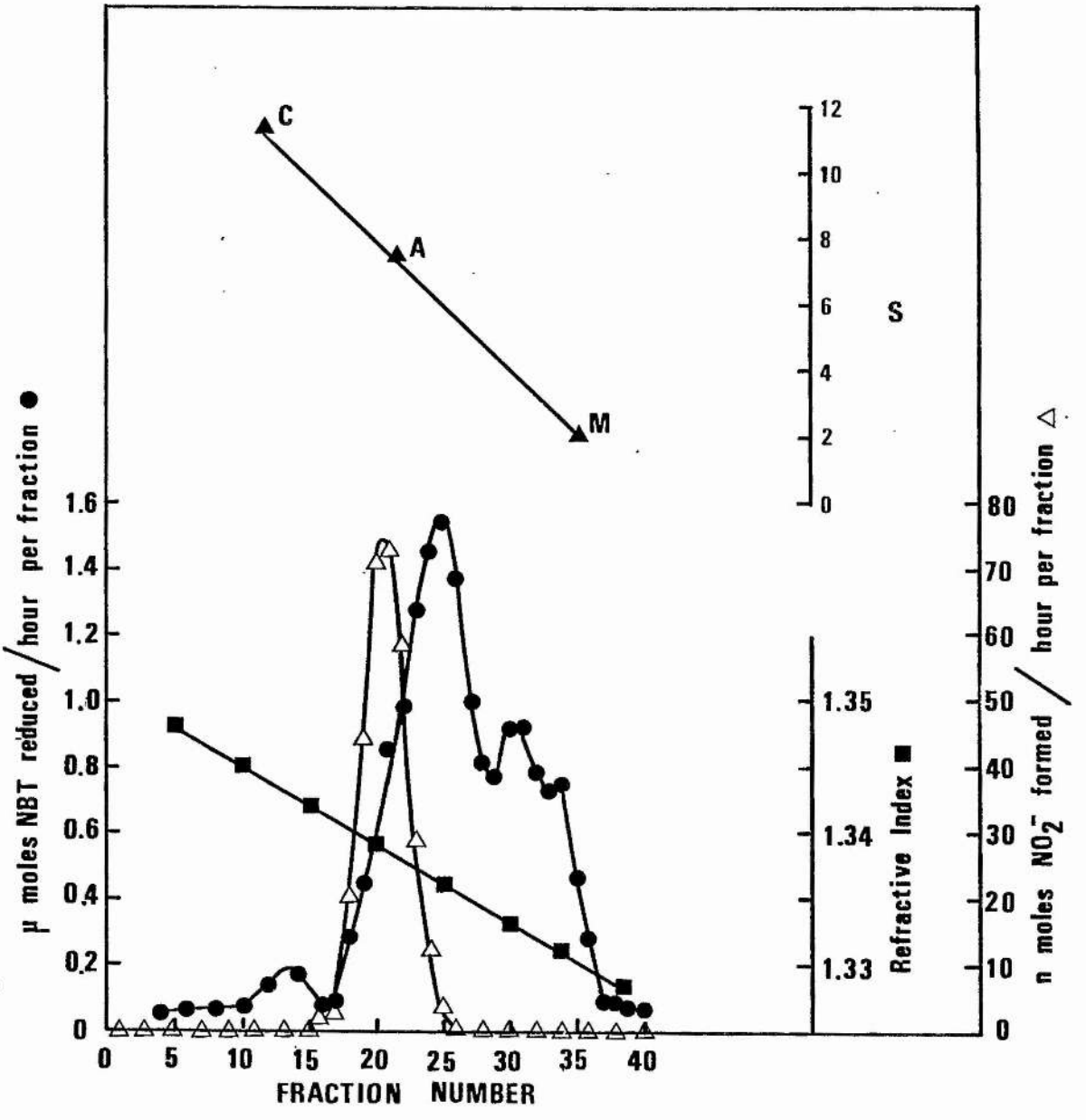

C
A
M
12
10
8
6
S
4
2
0
2.5
2.0
1.5
1.0
0.5
0
1.35
1.34
1.33
Refractive Index ■
10
8
6
4
2
0
μ moles cyt c reduced / hour per fraction △
μ moles DCPIP reduced / hour per fraction ●
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

FIG. 54

Distribution of NADH-Nitrate Reductase and NADH-NBT Reductase Activities in Extracts from 90-hour old Nitrate Plants

This figure shows the distribution of NADH-nitrate reductase and NADH-NBT reductase activities following sucrose density gradient centrifugation of an extract from 90-hour old nitrate plants. C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


C
A
M
12
10
8
6
S
4
2
0
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
μ moles NBT reduced / hour per fraction ●
1.35
1.34
1.33
Refractive Index ■
80
70
60
50
40
30
20
10
0
n moles NO_2^- formed / hour per fraction △
0 5 10 15 20 25 30 35 40
FRACTION NUMBER

major peaks of activity were detected, corresponding to sedimentation coefficients of 3.3 S, 6.2 S and 11 S of which the 6.2 S peak is much the largest.

The data using ferricyanide as substrate has not been presented due to difficulties encountered when assaying fractions from the sucrose gradient. Although linear rates had been found when developing the assay method with crude extracts, when fractions from the sucrose gradient were assayed biphasic rates were found, making interpretation of the data rather difficult. However, as was found when DCPIP and NBT were used as substrates, it was evident that no peak of NADH-ferricyanide reductase activity was associated with that of NADH-nitrate reductase activity. Apparent peaks of NADH-ferricyanide activity were found to have sedimentation coefficients of 3 S, 4.4 S, 5.2 S and 6.4 S.

It may therefore be concluded that cytochrome c is unique among the dehydrogenase substrates tested in that it is the only one to show a peak of activity coincident with that of NADH-nitrate reductase activity following sucrose density gradient centrifugation of an unpurified extract from nitrate plants. This strongly suggests that results using the alternative dehydrogenase substrates with relatively impure samples of higher plant nitrate reductase should be regarded with extreme caution as although these substrates may be able to accept electrons from nitrate reductase, they do so at a rate much lower than the rates at which they accept electrons from other

enzymes extracted from barley.

SECTION II - CAN THE ALTERNATIVE DEHYDROGENASE SUBSTRATES INHIBIT NADH-NITRATE REDUCTASE ACTIVITY?

If a compound can accept electrons from nitrate reductase then it should be possible to demonstrate a competition for electrons between that compound and nitrate (Solomonson and Vennesland, 1972) because the electrons would be channelled to the dehydrogenase substrate rather than through to nitrate. The ability of each of the alternative dehydrogenase substrates to compete with nitrate for electrons from NADH was therefore tested.

It is essential to have a high NADH concentration for these experiments to counteract the demand by other NADH-dehydrogenases which may be present and which are also capable of donating electrons to the substrate under test. The NADH concentration used for these experiments was 0.24 mM, which is twice the concentration used in the standard NADH-nitrate reductase assay and is approximately 50-fold greater than the K_M for NADH of higher plant nitrate reductases (Eaglesham and Hewitt, 1975; Campbell and Smarrelli, 1978).

The nitrate reductase sample used in these experiments had been approximately 12-fold purified by streptomycin sulphate treatment, $(NH_4)_2SO_4$ fractionation and gel filtration through Biogel A1.5 m. The peak fractions from the gel

filtration step were used and each dehydrogenase substrate was tested at a concentration of 0.1 mM. The level of inhibition found with each of these substrates is shown below in Table 17.

TABLE 17

Dehydrogenase Substrate	% Inhibition
Cytochrome c	93
DCPIP	100
NBT	90
Ferricyanide	61

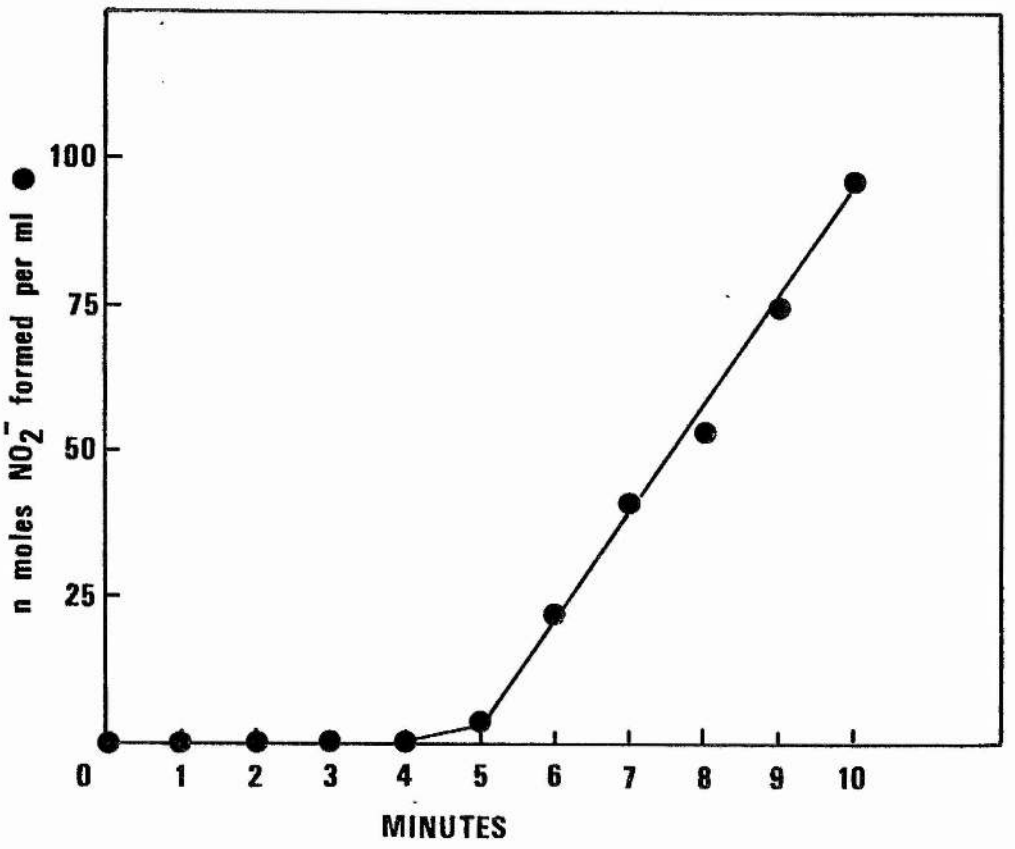
These results demonstrate that each of the dehydrogenase substrates tested is capable of competing with nitrate for electrons from NADH and suggest therefore that each is capable of accepting electrons from nitrate reductase.

The ability of DCPIP to act as a substrate for nitrate reductase was also shown by a different method. By including 10 μM DCPIP in the NADH-nitrate reductase assay, it was possible to demonstrate a lag, during which the DCPIP was reduced, following which nitrite production could be detected (Fig. 55). This result rules out the possibility that depletion of NADH is responsible for the inhibitions of nitrate reductase activity observed in the

FIG. 55

Effect of 10 μM DCPIP on NADH-Nitrate Reductase Activity

This figure shows that the presence of 10 μM DCPIP results in a lag in the production of nitrite by nitrate reductase. Nitrite production commences when all the DCPIP has been reduced by nitrate reductase.


n moles NO_2^- formed per ml
100
75
50
25
0
1
2
3
4
5
6
7
8
9
10
MINUTES

presence of the dehydrogenase substrates.

SECTION III - CAN THE DEHYDROGENASE SUBSTRATES ACT AS SUBSTRATES FOR PURIFIED NITRATE REDUCTASE?

The ability of these dehydrogenase electron acceptors to act as a substrate for nitrate reductase was further tested using 550-fold purified nitrate reductase. This was obtained from the purification reported in Section VI of Chapter 1 of these Results. Aliquots from the peak fraction (Fig. 21) following Blue Dextran-Sepharose chromatography were subjected to sucrose density gradient centrifugation as described in Methods, Section IV. The results are presented below.

With Cytochrome c as Substrate

The distribution of NADH-cytochrome c reductase activity following sucrose density centrifugation of highly purified nitrate reductase is presented in Fig. 56a. It is evident that two species of NADH-cytochrome c reductase are present, one cosedimenting with NADH-nitrate reductase activity (Fig. 56b) at 7.7 S and the other with a sedimentation coefficient of 3.1 S. This is most likely to be the 40 000 molecular weight NADH-cytochrome c reductase species previously described in Chapters 2 and 3 of these Results.

It has previously been suggested (Chapter 3) from poly-

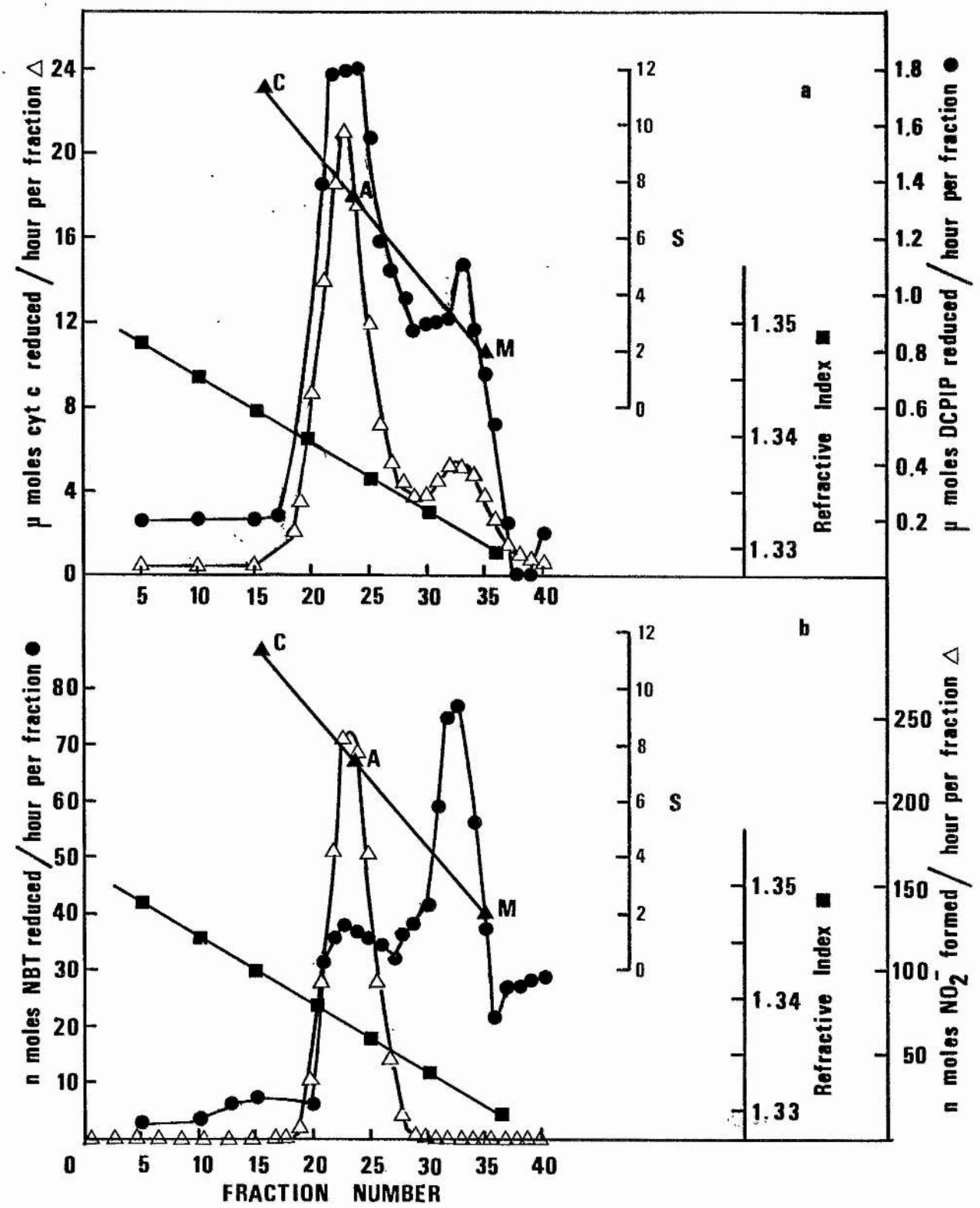
FIG. 56

Correlation of NADH-Dehydrogenase Activities with that of Purified Nitrate Reductase

Fig. 56a shows the distribution of NADH-cytochrome c reductase and NADH-DCPIP reductase activities following sucrose density gradient centrifugation of a sample of purified barley nitrate reductase.

Fig. 56b shows the distribution of NADH-nitrate reductase and NADH-NBT reductase activities following sucrose density gradient centrifugation of a sample of purified barley nitrate reductase.

C, A and M denote the positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin, respectively. Sedimentation is from right to left.


a
b
C
A
M
S
Refractive Index ■
1.35
1.34
1.33
μ moles cyt c reduced / hour per fraction △
μ moles DCPIP reduced / hour per fraction ●
n moles NBT reduced / hour per fraction ●
n moles NO_2^- formed / hour per fraction △
FRACTION NUMBER

acrylamide gel electrophoresis of both the same purified nitrate reductase sample used in this experiment and the purified 40 000 molecular weight species, that this species was a contaminant of the purified nitrate reductase sample. The results presented here therefore confirm this suggestion. The presence of the 40 000 molecular weight NADH-cytochrome c reductase, which has been shown previously to be derived from nitrate reductase, allows the ability of DCPIP and NBT to act as substrates for this protein to be determined in addition to their role as substrates for nitrate reductase.

With DCPIP as Substrate

The distribution of NADH-DCPIP reductase activity following sucrose density gradient centrifugation of purified nitrate reductase is presented in Fig. 56a. In contrast to the results obtained when a crude extract was subjected to the same technique (Fig. 53), peaks of NADH-DCPIP reductase activity were found to coincide with both peaks of NADH-cytochrome c reductase activity. It may therefore be concluded that DCPIP is capable of acting as a substrate for both nitrate reductase and the 40 000 molecular weight NADH-cytochrome c reductase species.

It is possible, by comparing the peak rates of reduction of both cytochrome c and DCPIP by nitrate reductase, to comment on their relative effectiveness as substrates for the dehydrogenase component of the enzyme. The maximum

activity observed with cytochrome c as substrate was 20.8 µmoles reduced per hour whereas with DCPIP as substrate, the maximum was only 1.7 µmoles reduced per hour. Even allowing for the slight flattening of the peak of NADH-DCPIP reductase activity (Fig. 56a) this value is still about 10-fold lower than that obtained with cytochrome c as substrate.

With NBT as Substrate

The distribution of NADH-NBT reductase activity following sucrose density gradient centrifugation of purified nitrate reductase is presented in Fig. 56b. It is immediately obvious that the major peak of activity is not associated with that of NADH-nitrate reductase activity (Fig. 56b) but instead corresponds to the 40 000 molecular weight NADH-cytochrome c reductase evident in Fig. 56a. Although there is a broad peak of NADH-NBT reductase corresponding to the peak of NADH-nitrate reductase, the peak height is only half of that associated with the slower sedimenting species.

It may therefore be concluded that although NBT is capable of acting as a substrate for the dehydrogenase activity of nitrate reductase, it only does so very poorly and with a maximum activity about 550-fold lower than that obtained with cytochrome c as substrate. In contrast, the NADH-NBT reductase activity associated with the 40 000 molecular weight NADH-cytochrome c reductase species was 'only' 65-fold lower than that observed with cytochrome c as substrate.

It was not possible to evaluate the ability of ferricyanide to accept electrons from purified nitrate reductase as time did not permit the development of an alternative method of assay in order to prevent biphasic rates from being detected.

DISCUSSION

It may be concluded from the results presented in this Chapter that the assumptions made by Hewitt (1975) and others are correct and that both DCPIP and NBT are capable of acting as substrates for the dehydrogenase activity of higher plant nitrate reductase. It would also seem likely from the limited data presented, that ferricyanide can also act as a substrate for this activity.

It is, however, evident that cytochrome c is a much better substrate for nitrate reductase than any of the alternative electron acceptors tested here. If the rate of reduction of cytochrome c by nitrate reductase is expressed as 100%, then the rate of reduction of DCPIP is only 10% whilst the rate of reduction of NBT is only about 0.2%. These data presumably reflect the ease with which these substrates can accept electrons from nitrate reductase and are therefore surprising as both DCPIP and NBT (with molecular weights of less than 300) would be expected to have much greater access to the dehydrogenase site than the much larger cytochrome c molecule which has a molecular weight of approximately 12 000. Thus, the structural requirements of the dehydrogenase site (or sites?) would seem to greatly

favour the structure of cytochrome c.

The presence of the 40 000 molecular weight NADH-cytochrome c reductase species following sucrose density gradient centrifugation of purified nitrate reductase allows similar calculations to be undertaken for this species which has been shown to be derived from nitrate reductase. It is noticeable that, although cytochrome c was still the most effective substrate, the relative rates with DCPIP (21%) and NBT (1.5%) were much higher than those observed for nitrate reductase. Thus DCPIP is 3-fold and NBT is 8-fold more effective as a substrate for the 40 000 molecular weight NADH-cytochrome c reductase than as a substrate for the dehydrogenase activity of nitrate reductase. As the protein moiety is likely to be the same in each case it would appear likely that these changes in effectiveness reflect changes in protein conformation around the dehydrogenase site.

Recently, Maldonado, Notton and Hewitt (1978a) have attempted to determine the sites of interaction of some of the dehydrogenase substrates with spinach nitrate reductase. Their kinetic studies were performed with partially purified enzyme (specific-activity not reported) and suggested that while DCPIP was only capable of interacting at the dehydrogenase site of nitrate reductase, ferricyanide could interact both at this site and also the nitrate-reducing site of the enzyme.

An inhibition of nitrate-binding by ferrocytochrome c

was also reported but was only functional when NADH was used as electron donor. Subsequently, the same authors (Maldonado, Notton and Hewitt, 1978b) demonstrated a synergistic effect between NADH and ferrocytochrome c which led to inactivation of the spinach nitrate reductase. This prompted the authors to suggest that ferrocytochrome c was involved in the *in vivo* regulation of higher plant nitrate reductase although no suggestions were made as to the form this would take.

Thus the very high affinity of barley nitrate reductase for cytochrome c which is evident even in analysis of crude extracts (Fig. 53) may be a reflection of the possible physiological role played by cytochrome c in the regulation of nitrate reductase. However, it would appear likely that the regulatory site for ferrocytochrome c (should it exist) would be quite separate from the dehydrogenase site at which cytochrome c interacts. Further work is required in order to clarify this issue as insufficient data is available to allow the observations to be evaluated properly.

CHAPTER 6

REVERSIBLE INACTIVATION AND PHOTOREACTIVATION OF BARLEY NITRATE REDUCTASE

INTRODUCTION

The key position held by nitrate reductase in the assimilation of nitrate by green algae and higher plants has led many workers to study the regulation of this enzyme in an effort not only to understand the physical nature of nitrate reductase, but also to examine the regulation of nitrate assimilation as a whole. As indicated in the Introduction to this thesis, whereas fungal nitrate reductases appear to be regulated at the level of enzyme synthesis, the nitrate reductases from algae and higher plants appear to be regulated rather by a system of reversible inactivation, which is described below.

(a) In Green Algae

Early studies with *Chlorella* (Syrett and Morris, 1963; Morris and Syrett, 1963) showed that growth in the presence of ammonium led to repression of nitrate reductase synthesis and inactivation of nitrate reductase. Losada *et al.* (1970) later confirmed that ammonium could inactivate nitrate reductase activity but showed that the NADH-cytochrome c reductase (= diaphorase) activity was unaffected. In both *Chlorella* (Moreno *et al.*, 1972) and *Chlamydomonas* (Herrera *et al.*, 1972) it was demonstrated that the effect of ammonium was to uncouple noncyclic photophosphorylation and the consequent increase in NADH concentration was shown to directly inhibit nitrate reductase activity. These results were subsequently confirmed for both species

(Losada *et al.*, 1973; Caparro *et al.*, 1976).

It was also shown that NADH-inactivated nitrate reductase could be reactivated by the addition of either nitrate (Vennesland and Jetschmann, 1971) or ferricyanide (Jetschmann, Solomonson and Vennesland, 1972) and this led to the discovery that nitrate reductase from *Chlorella vulgaris* is extracted in a partially inactive form even when the cells are grown on nitrate.

In contrast to the results with *C. vulgaris* nitrate reductase, Maldonado *et al.* (1973) reported that *C. fusca* nitrate reductase could not be inactivated with NADH alone, but required the presence of ADP, which prompted the authors to suggest that ADP was an important cofactor in the regulation of nitrate reductase.

Solomonson (1974) noted a similar co-operative effect of NADH and cyanide to inactivate *C. vulgaris* nitrate reductase, which could be reactivated with ferricyanide if the excess NADH and cyanide were removed. There was no change in absorption spectrum, molecular size or oxidation state of the cyt b_{557} component during reactivation. The presence of HCN was demonstrated in *C. vulgaris* by Gewitz *et al.* (1974) who suggested that cyanide might be involved in the *in vivo* regulation of nitrate reductase. Further support for this hypothesis was obtained by Lorimer *et al.* (1974) who reported the firm binding of $^{14}CN^-$ to nitrate reductase following inactivation. The same amount of $^{14}CN^-$ was bound by either *in vitro* or *in vivo* inactivation.

Identical results were later obtained with the nitrate reductase from *C. fusca* by Gewitz, Piefke and Vennesland (1978). These results strongly suggest that algal nitrate reductase is regulated by a reversible inactivation mechanism which involves cyanide.

(b) In Higher Plants

Much less work has been done on the regulation of higher plant nitrate reductase but spinach nitrate reductase can be inactivated with NADH (Palacian *et al.*, 1974; de la Rosa *et al.*, 1976) and a co-operative effect with cyanide has also been shown (Aparicio, Roldan and Calero, 1976). Spinach nitrate reductase can be reactivated with either nitrate or ferricyanide (Palacian *et al.*, 1974) as well as with riboflavin, FMN or FAD (de la Rosa *et al.*, 1976). This flavin-dependent reactivation of spinach nitrate reductase has recently been shown to be strongly light-dependent with a specific enhancement by blue light (Aparicio, Roldan and Calero, 1976; Roldan, Calero and Aparicio, 1978; Aparicio and Maldonado, 1979).

Very recently, rice nitrate-reductase has been shown (Leong and Shen, 1979) to be inactivated by NADH and cyanide, and reactivated with ferricyanide. In contrast to other workers, these authors also found that the rice enzyme was automatically reactivated by the removal of NADH and cyanide.

It was therefore decided to test the ability of barley nitrate reductase to undergo reversible inactivation and photoreactivation. If successful, these experiments may indicate that this form of regulation is a general phenomenon (at least *in vitro*) of higher plant nitrate reductases.

RESULTS

SECTION I - INACTIVATION OF BARLEY NITRATE REDUCTASE

Barley nitrate reductase was purified from 200 g of barley shoots by streptomycin sulphate treatment, $(NH_4)_2SO_4$ fractionation at 45% saturation, and Biogel A1.5 m gel filtration, all as described in Methods, Section II. The peak fractions of nitrate reductase activity were pooled and the protein precipitated with 50% $(NH_4)_2SO_4$ and dissolved in a small volume of 0.05 M potassium phosphate buffer, pH 7.5 (lacking EDTA, FAD and cysteine).

Aliquots of the enzyme were then inactivated by the addition of NADH and cyanide and all containers were wrapped in foil to exclude light and thereby prevent any photoreactivation due to bound flavin (Roldan, Calero and Aparicio, 1978). The results of a preliminary inactivation are given in Table 18.

The results obtained by the addition of 1 mM NADH were very unexpected. Contrary to the reports with spinach nitrate reductase (Palacian *et al.*, 1974), NADH was found to activate barley nitrate reductase, rather than

TABLE 18

Additions*	Inhibition (%)
None	0
1 mM NADH	+28
0.3 mM KCN	77
1 mM NADH + 0.3 mM KCN	95

* All additives were present for two minutes prior to assay. All concentrations are final concentrations

\+ denotes activation

inhibit it. There is one previous report of a similar result, obtained with nitrate reductase from rice shoots (Gandhi, Sawhney and Naik, 1973). NADH was also found to protect rice nitrate reductase from inactivation by a factor isolated from rice roots (Kadam *et al.*, 1974) but as no such factor could be detected in rice shoots, the previous observations remain unexplained.

As found by many other workers, cyanide caused a marked inactivation (77%) of barley nitrate reductase activity whilst the presence of both NADH and cyanide resulted in almost total (95%) inactivation of barley nitrate reductase within two minutes. An equally effective and rapid inactivation of barley nitrate reductase was also achieved with lower concentrations of these reagents - 0.8 mM NADH and 0.17 mM KCN.

In order to remove excess NADH and cyanide, this latter sample (inactivated with 0.85 mM NADH and 0.17 mM KCN) was passed through a column (1.5 cm × 20 cm) of Sephadex G25 previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. Both the column and the resultant fractions were wrapped in foil to exclude light and the inactivated nitrate reductase detected by the absorbance at 280 nm of the protein in the sample. Protein-containing fractions were pooled and then divided into aliquots for reactivation of the nitrate reductase.

SECTION II - REACTIVATION OF BARLEY NITRATE REDUCTASE

For reactivation of the nitrate reductase activity, the different aliquots were subjected to the treatments described below in Table 19.

TABLE 19

Treatment	% Reactivation
Dark + no additions	15.4
Light* + no additions	22.2
Dark + 1 mM ferricyanide	64.6
Light* + 1 mM ferricyanide	44.8
Dark + 100 µM FAD	18.4
Light* + 100 µM FAD	72.8

* Light was supplied by six white fluorescent tubes for 10 minutes prior to assay.
All concentrations are final concentrations.

Thus it is clear that inactivated barley nitrate reductase, maintained in darkness following removal of free NADH and cyanide, undergoes only a slight reactivation. Exposure to light marginally increases the level of reactivation presumably due to the presence of bound flavin on the enzyme. As expected, the presence of 1 mM ferricyanide resulted in a very marked reactivation of barley nitrate reductase. However, the reason for the extent of this reactivation being 20% greater in the dark than in the light is not clear, as no similar effect has been reported in the literature. The presence of 100 µM FAD had almost no effect on the sample maintained in darkness but the effect of light and FAD was to produce the highest level of reactivation of the methods tested. Thus it has been clearly demonstrated that barley nitrate reductase can be inactivated, reactivated and photoreactivated.

DISCUSSION

The results presented in this chapter have shown that, with the exception of the apparent activation by NADH, barley nitrate reductase exhibits all the major phenomena of reversible inactivation and photoreactivation reported for the nitrate reductases from spinach and green algae. It is probable that the level of reactivation observed with ferricyanide is lower than that observed with FAD in the light because a relatively high concentration of ferricyanide was used and concentration is known to affect the

percentage reactivation (Leong and Shen, 1979). These authors reported that rice nitrate reductase was automatically reactivated after removal of NADH and cyanide by Sephadex G25 filtration. However, analysis of their method reveals that 5 μM FAD was present throughout and so it would appear likely that the 'automatic' reactivation was due to photoreactivation of the enzyme in the presence of FAD.

The mechanism of reactivation of nitrate reductase by ferricyanide has recently been elucidated. As ferri cyanide is an electron acceptor for the dehydrogenase activity of nitrate reductase (Hewitt, 1975), reactivation can either occur by back electron flow from Mo to the dehydrogenase site or, possibly, by direct oxidation of Mo (Hewitt, 1975). Funkhouser and Ackerman (1976) showed that millimolar concentrations of manganese pyrophosphate could partially reactivate *C. vulgaris* nitrate reductase but was not a substrate for the dehydrogenase activity of the enzyme. Thus, reactivation could not be due to back electron flow to the dehydrogenase site. Conversely, ferric-o-phenanthroline was shown by the same authors to be a very good dehydrogenase substrate, but failed to reactivate the inactive enzyme.

The kinetic studies of Maldonado, Notton and Hewitt (1978a) showed that ferrocyanide consistently altered nitrate binding to spinach nitrate reductase for all three classes of reductant (NADH, FMNH and reduced methyl

viologen) and they concluded that ferricyanide could react directly with the Mo site of nitrate reductase and thereby cause reactivation. These authors also speculated that the naturally-occurring oxidant for reactivation of spinach nitrate reductase might be Mn, deficiency of which leads to substantial nitrate accumulation in plants (Hewitt, 1963). This is in conflict with the results of Pistorius *et al.* (1976) who showed that Mn-deficient *C. vulgaris* cells had an impaired ability to inactivate nitrate reductase.

A model for the regulation of nitrate assimilation in photosynthetic tissues has been recently presented (Solomonson and Spehar, 1977; Solomonson, 1978) in which CO_2 fixation and nitrate assimilation are co-ordinately controlled by the intracellular ratio of O_2 to CO_2. This model accounts for the observed effects of O_2, CO_2 and light on nitrate assimilation and provides a rationale for the *in vivo* regulation of nitrate reductase by cyanide. In this model, hydroxylamine, a possible intermediate in the reduction of nitrite to ammonium, reacts with glyoxylate to form glyoxylate oxime which is in turn converted to CN^- and CO_2. Thus, cyanide could be derived from phosphoglycollate, a product of RuBP oxygenase.

The model assumes that hydroxylamine is an endogenous metabolite and support for this has been recently presented (Loussaert and Hageman, 1976), in contrast to previous results from the same laboratory (Beevers and Hageman, 1972). In extracts from *C. vulgaris* cyanide formation has

been demonstrated from hydroxylamine and glyoxylate oxime (Solomonson and Spehar, 1977) or from just glyoxylate oxime (Solomonson and Spehar, 1979). Cyanide formation was shown to be dependent on Mn^{2+} ions, supporting the observation of Pistorius *et al.* (1976) that Mn-deficient *C. vulgaris* cells displayed an impaired ability to inactivate nitrate reductase. Cyanide formation has also been shown to be stimulated by as much as 20-fold by ADP (Spehar and Solomonson, 1978; Solomonson and Spehar, 1979) and these authors suggest that ADP may therefore be important in the regulation of nitrate reductase due to its effect on cyanide formation rather than to a direct effect on nitrate reductase, as had previously been suggested (Maldonado *et al.*, 1973).

Recent work (Okabe, Codd and Stewart, 1979) with the blue-green alga *Anabaena cylindrica* has cast some doubt on the above model. These authors demonstrated a stimulation of RuBP carboxylase and an inhibition of RuBP oxygenase by hydroxylamine. A similar stimulation had been previously observed in spinach (Bhagwat, Ramakrishna and Sane, 1978) but no inhibition of RuBP oxygenase was detected. Okabe, Codd and Stewart (1979) have pointed out that although cyanide production of *C. vulgaris* has been demonstrated (Gewitz *et al.*, 1976), the cyanide-inactivation of *C. variegata* nitrate reductase does not occur when nitrate is present (Hipkin, Al-Bassam and Syrett, 1978). The direct inhibition of RuBP oxygenase by hydroxylamine suggests

that regulation of photorespiratory carbon flow and nitrate assimilation may occur without the involvement of cyanide (Okabe, Codd and Stewart, 1979).

As indicated in the Introduction to this Chapter, inactivation of nitrate reductase with NADH and cyanide does not affect the dehydrogenase activity of the enzyme. It is possible to inactivate this portion of nitrate reductase by treatment with sulphydryl-group reagents, e.g. pCMB (Schrader *et al.*, 1968). The enzyme still retains the ability to reduce nitrate with either FMNH or reduced methyl viologen following pCMB treatment, and these activities can still be inactivated by treatment with NADH (Castillo, de la Rosa and Palacian, 1975) indicating that a functional dehydrogenase activity is not required for the NADH-inactivation of nitrate reductase.

The NADH-inactivation of FMNH-nitrate reductase has an FAD-requirement following pCMB treatment (Castillo *et al.*, 1976) but although spinach nitrate reductase is only catalytically active with NADH, both NADH and NADPH are active in causing inactivation. These authors also found that the FMNH-nitrate reductase activity could not be inactivated by NADH if the NADH-dehydrogenase activity had first been destroyed by heat-inactivation in the absence of FAD, in contrast to the results obtained with pCMB. It has subsequently been shown that the dehydrogenase activity of pCMB-treated nitrate reductase can be reactivated by the addition of FAD (Castillo, de la Rosa and Palacian, 1977).

Thus it is clear that there are many ways in which higher plant and algal nitrate reductases may be regulated. The relative importance and interaction of these will only be elucidated by a continuation of the vast amount of work already undertaken in this field.

CHAPTER 7

HEAT INACTIVATION STUDIES ON BARLEY NITRATE REDUCTASE

INTRODUCTION

Higher plant nitrate reductase has associated with it three additional partial activities-NADH-cytochrome c reductase, FMNH-nitrate reductase and reduced methyl viologen nitrate reductase - which presumably reflect either the subunit composition of the enzyme or differing 'functional regions' of the enzyme. It is therefore possible to probe the physical nature of nitrate reductase by determining the effect of an additive or treatment upon the partial activities of nitrate reductase. In this way it has been possible to show that the NADH-dehydrogenase activities of nitrate reductase are affected by sulphydryl reagents whilst the FMNH- and reduced viologen-dependent activities are not (Schrader *et al.*, 1968). In a similar manner, the nitrate-reducing activities can all be inactivated by azide or cyanide (Solomonson and Vennesland, 1972; Garrett and Greenbaum, 1973) whilst the NADH-dehydrogenase activities are unaffected.

Heat-inactivation has also been used to probe the structure of nitrate reductase. Wray and Filner (1970) have shown that the NADH-dependent activities of barley nitrate reductase have very short half-lives at 45°C whereas the FMNH-nitrate reductase activity was relatively heat stable. The thermal stability of reduced methyl viologen-nitrate reductase activity was not tested by these authors.

All these results indicate that nitrate reductase has

two functional regions, an NADH-oxidising region responsible for the NADH-dehydrogenase activities and a nitrate reducing region, to which both FMNH and reduced methyl viologen donate electrons.

As indicated in the Introduction to this thesis, reduced viologens are believed to donate electrons directly to the Mo site of nitrate reductase (Hewitt, Hucklesby and Notton, 1976) but the site of interaction of FMNH is not known.

If it were possible to demonstrate different thermal stabilities for FMNH- and reduced viologen-dependent nitrate reductase activities then it could be concluded that these reductants were capable of interacting at differing sites on the enzyme and might point to the existence of a third 'functional region' of the nitrate reductase molecule. It was therefore decided to repeat the work of Wray and Filner (1970), analysing all the partial activities associated with barley nitrate reductase.

RESULTS

Nitrate reductase was partially purified from 250 g of barley shoots by treatment with streptomycin sulphate, $(NH_4)_2SO_4$ fractionation at 45% saturation and gel filtration through Biogel A1.5 m, all steps as described in Methods, Section II. Fractions containing peak levels of nitrate reductase activity were pooled and divided into

2 ml aliquots which were then placed in a water bath at 45°C. After the appropriate time intervals, each aliquot was removed and the activity of all the nitrate reductase-associated reactions then determined for each aliquot (Fig. 57).

SECTION I - THE NADH-DEPENDENT ACTIVITIES

NADH-nitrate reductase was found to be the least stable of all the activities tested, as was previously found by Wray and Filner (1970). Analysis of the data (Fig. 57) by linear regression yielded a half-life for NADH-nitrate reductase activity of only 3.38 minutes at 45°C. NADH-cytochrome c reductase activity was almost equally unstable, with a half-life of only 4.08 minutes at 45°C. Had the stabilities of each of these activities depended on exactly the same region of the nitrate reductase molecule, then these two half-lives should have been the same. The possible reasons for the small, but significant, difference between the two will be examined in the Discussion at the end of this Chapter.

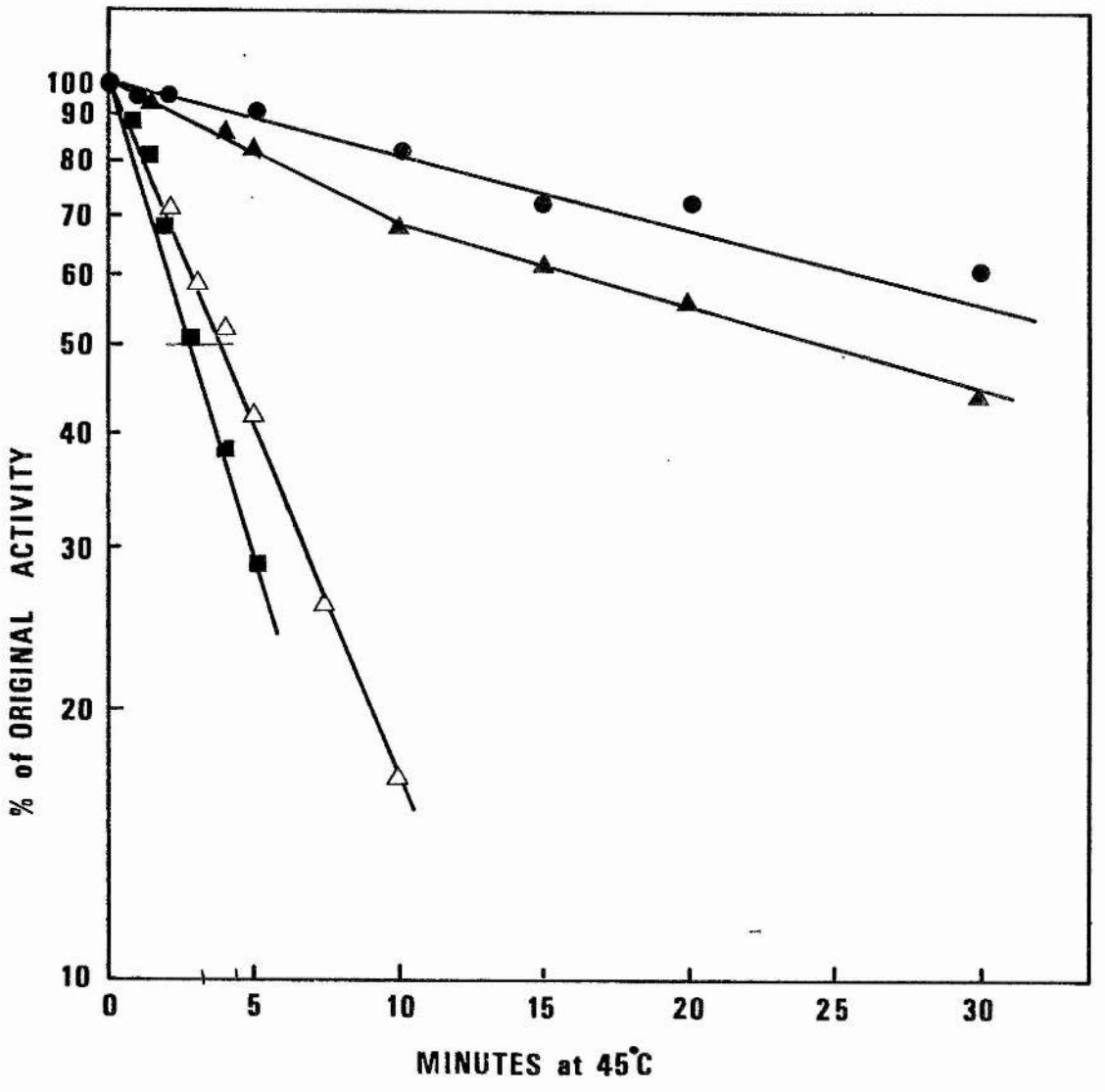
SECTION II - FMNH- AND REDUCED VIOLOGEN-DEPENDENT ACTIVITIES

In contrast to the NADH-dependent activities, the stability of both FMNH- and reduced methyl viologen-nitrate reductase activities were much greater. Linear regression analysis of the data yielded half-lives at 45°C of 23.59 minutes for FMNH-nitrate reductase and 34.98 minutes for

FIG. 57

Thermal Inactivation of Nitrate Reductase-Associated Activities

This figure shows the rates of thermal inactivation of NADH-nitrate reductase activity (■—■), NADH-cytochrome c reductase activity (△—△), FMNH-nitrate reductase activity (▲—▲) and reduced methyl viologen-nitrate reductase activity (●—●) during incubation at 45°C. Experimental details are given in the main text.


100
90
80
70
60
50
40
30
20
10
% of ORIGINAL ACTIVITY
0
5
10
15
20
25
30
MINUTES at 45°C

reduced methyl viologen nitrate reductase activity, suggesting that the two reductants are capable of donating electrons to separate sites on the nitrate reductase molecule (Wray, Small and Brown, 1979).

However, another interpretation of the data is possible and Fig. 57 has been drawn to emphasise the apparent biphasic nature of the thermal stability of FMNH-nitrate reductase. During the first 10 minutes at 45°C, the rate of inactivation of FMNH-nitrate reductase is intermediate between that of the NADH-dependent activities and that of reduced methyl viologen nitrate reductase activity. After 10 minutes at 45°C the subsequent rate of inactivation of FMNH-nitrate reductase activity is almost identical to the rate of inactivation of reduced methyl viologen nitrate reductase activity indicating that both reductants may be donating electrons to the same region of nitrate reductase.

It is possible to calculate that during the first 10 minutes at 45°C the difference in the rate of inactivation of FMNH-nitrate reductase compared to that of reduced methyl viologen nitrate reductase could be equivalent to a contribution of 17.5% of the rate observed for the NADH-dependent activities. This suggests that FMNH is capable of donating electrons to nitrate reductase at two separate sites, one of which is close to the dehydrogenase site of the enzyme while the other is very close to that used by reduced methyl viologen. The data (Fig. 57) suggest that this latter site is responsible for about 80% of the

observed FMNH-nitrate reductase activity.

DISCUSSION

The thermal stabilities reported here for the activities associated with barley nitrate reductase differ markedly from those reported by Wray and Filner (1970) who reported half-lives of inactivation of 0.5 minutes for NADH-nitrate reductase activity and 1.5 minutes for NADH-cytochrome c reductase activity (compared with 3.38 minutes and 4.08 minutes reported here). This difference is probably due to the presence of FAD in the buffers used here as FAD has been shown to protect against heat inactivation of the nitrate reductases from *Chlorella* (Zumft *et al.*, 1970), spinach (Relimpio *et al.*, 1971; Palacian *et al.*, 1974) and maize (Roustan, Neuberger and Fourcy, 1974).

The large discrepancy in half-lives between the two NADH-dependent activities reported by Wray and Filner (1970) is likely to be due to the use by those authors of a crude extract from barley which would contain several NADH-cytochrome c reductase species. In the work reported here, partially purified barley nitrate reductase was used in order to exclude the other species of NADH-cytochrome c reductase activity, but a small difference between the thermal stabilities of the two NADH-dependent activities was still evident (Fig. 57). The fact that NADH-nitrate reductase is the less stable of the two activities indicates

that conformational changes probably occur upon exposure to higher temperatures (45°C), such that electron transfer between the NADH-oxidising moiety of the enzyme and the nitrate reducing moiety of the enzyme is prevented, despite the fact that the individual activities expressed by those regions of the molecule are still functional. It is also possible that treatment at 45°C results in breakdown of nitrate reductase, abolishing the overall NADH-nitrate reductase activity whilst the remaining activities can still be expressed by the released fragments. This would, however, appear unlikely as Wray and Filner (1970) have demonstrated a clear peak of FMNH-nitrate reductase activity, with the same sedimentation coefficient as NADH-nitrate reductase from untreated extract, following sucrose density gradient centrifugation of heat-inactivated nitrate reductase.

The possibility that FMNH may donate electrons at two different sites on nitrate reductase, as suggested by the results presented here, can be supported by some results obtained with *N. crassa* nitrate reductase (Garrett and Nason, 1969). During heat-inactivation of this enzyme at 49°C the dehydrogenase activities lose above 90% of their activity within 3 minutes whilst FADH-nitrate reductase activity undergoes an initial 20% drop in activity following which it remains stable for at least 30 minutes. Reduced viologen-nitrate reductase activity shows an initial increase following which it too is stable for

at least 30 minutes. These results may be interpreted to indicate that the initial 20% reduction in FADH-nitrate reductase activity is due to the heat inactivation of the dehydrogenase site, to which it donates 20% of its electrons. The stability of the remaining activity would parallel that observed for reduced viologen-nitrate reductase activity because both reductants are donating electrons to the same functional region of nitrate reductase.

Additional support for this hypothesis comes from the observation (I. Small, unpublished) that the presence of 50 μM reduced DCPIP causes a 20% inhibition of barley FMNH-nitrate reductase activity. Maldonado, Notton and Hewitt (1978a) have indicated that DCPIP is only capable of interacting at the dehydrogenase site of nitrate reductase and so it may be concluded that reduced DCPIP was competing with FMNH at the dehydrogenase site of the enzyme. These authors also indicated that reduced DCPIP interfered in nitrite estimation but it was found here that the presence of 50 μM reduced DCPIP in standard nitrite solutions caused only a 12% reduction in the dye intensity. The value of 20% inhibition of FMNH-nitrate reductase activity by 50 μM reduced DCPIP was corrected for this deviation in the assay.

It is therefore possible to conclude that there are probably two regions of the nitrate reductase molecule to which reduced flavins can donate electrons. One of these is at a site very close to the dehydrogenase site of the enzyme and the other is very close to the nitrate-reducing site of

the enzyme. It has not been possible to demonstrate whether this latter site is the same as that used by reduced methyl viologen with barley nitrate reductase but the results of Garrett and Nason (1969) with *N. crassa* nitrate reductase indicate that this is unlikely to be the case. These authors demonstrated that after 60 minutes at 55°C the FADH-nitrate reductase activity was lost whilst the reduced methyl viologen nitrate reductase activity was retained and was only destroyed after 100 minutes at 55°C or 1 minute at 68°C. These results strongly suggest that the site of electron donation by reduced viologen dyes is very close to the Mo site of nitrate reductase and probably closer to the Mo site than the point to which reduced flavins donate electrons. Insufficient data is available to allow the number of sites to which reduced viologen dyes donate electrons to nitrate reductase to be determined.

CHAPTER 8

ATTEMPTS TO DEMONSTRATE *IN VITRO* ASSEMBLY OF BARLEY NITRATE REDUCTASE

INTRODUCTION

As indicated in the Introduction to this thesis, *in vitro* reconstitution of *N. crassa* nitrate reductase has been performed by mixing extracts from induced *nit*-1 mycelia, which lack MCC, with MCC derived from a number of molybdoproteins by acid-treatment (Nason *et al.*, 1974). A similar *in vitro* reconstitution has been recently reported (Rucklidge, Notton and Hewitt, 1976; Hewitt, Notton and Rucklidge, 1977) with nitrate reductase from spinach. As no mutants of spinach nitrate reductase are available these authors grew spinach plants in the absence of Mo and extracts from these were used as source of apoprotein. MCC was prepared by acid-treatment of purified spinach nitrate reductase and when this was mixed with the extract from Mo-deficient spinach plants, nitrate reductase activity was reconstituted.

In the second of these two papers, the extracts from Mo-deficient spinach plants were subjected to sucrose density gradient centrifugation or gel filtration in order to separate the NADH-cytochrome c reductase species. Both the 8S and 3.7 S NADH-cytochrome c reductase species (equivalent to species B and C in Wray and Filner (1970), see Chapter 2 of these Results) were effective sources of apoprotein for the reconstitution of nitrate reductase activity, although the 3.7 S species was found to be more so.

As there have been no reports in the literature of a similar reconstitution using apoprotein from Mo-sufficient plants it was decided to attempt to demonstrate this.

RESULTS

SECTION I - PREPARATION OF MCC AND METHOD OF RECONSTITUTION

MCC was prepared by the method of Hewitt, Notton and Rucklidge (1977). Barley nitrate reductase was first partially purified by streptomycin sulphate treatment, $(NH_4)_2SO_4$ fractionation at 45% saturation and Biogel A1.5 m gel filtration, all steps as described in Methods, Section II. The most active fractions were pooled and the protein precipitated by adjustment to 50% saturation with $(NH_4)_2SO_4$, collected by centrifugation and then dissolved in a small volume of 0.15 M potassium phosphate buffer, pH 7.5.

This sample was placed on ice to cool and then acidified with 0.1 M HCl to pH 2-3 and allowed to stand at 0°C for 3-5 minutes. The pH was then readjusted to 7 with 0.1 M NaOH and the resulting precipitate immediately collected by centrifugation for 2 minutes in a bench top centrifuge and resuspended in ice-cold 0.1 M potassium phosphate buffer, pH 6.2. This was then mixed with the apoprotein under test, incubated at 20°C for 40 minutes to allow reconstitution, and then assayed for NADH-nitrate reductase activity.

The original authors (Hewitt, Notton and Rucklidge, 1977) reported that, provided the enzyme was contained in buffer of molarity of at least 0.1 M, acid-treatment completely abolished NADH-nitrate reductase activity. This was not found in the work reported here where residual activities ranging from 3.7% to 14.4% of the original activities were recovered following acid-treatment. Raising the buffer concentration to 0.3 M had no effect on these results. The results presented in the next Section have therefore been corrected for the residual NADH-nitrate reductase activity in the MCC preparation.

SECTION II - RECONSTITUTION OF NITRATE REDUCTASE ACTIVITY

Several attempts were made to reconstitute barley nitrate reductase activity using apoprotein derived either by sucrose density gradient centrifugation or Biogel filtration of an extract from nitrate plants (prepared as described in Methods, Section II). The results of these are presented in Table 20.

The initial experiments (numbers 1-3, Table 20) all used apoprotein derived by sucrose density gradient centrifugation of an extract from 90-hour old nitrate plants. Fractions containing the peak of NADH-cytochrome c reductase activity sedimenting between 2S and 4S (equivalent to species C in Wray and Filner, 1970; see Fig. 28) were pooled and an aliquot mixed with MCC. After incubation, the level of NADH-nitrate reductase activity reconstituted

TABLE 20

RECONSTITUTION OF BARLEY NADH-NITRATE REDUCTASE ACTIVITY *IN VITRO*

Experiment	Plant Age (Hours)	Method of Apoprotein Isolation	% Increase in Activity (Reconstitution/Controls)	Total Activity Reconstituted (nmoles NO_2^- formed/hour per ml)
1	90	a	151	66
2	90	a	11.6	32
3	90	a	0	0
4	90	b	29.4 - 55.3	15 - 17.25
5	90	b	40	20
6	144	b	-20	-
7	144	b	0*	0

[a] Sucrose Density Gradient Centrifugation

[b] Biogel A1.5 m Gel Filtration

* Reconstitution performed in the presence of BSA to counteract proteolysis.

was determined (Table 20, experiments 1-3) but the results were not found to be consistent, ranging from no reconstitution to an activity of 66 nmoles nitrite formed/hour per ml.

As this value is very low, an attempt was made to increase the amount of apoprotein, which may have been limiting in the above experiments. Samples corresponding to the same NADH-cytochrome c reductase species as used in the initial experiments were therefore isolated by Biogel A1.5 m gel filtration of an extract from 90-hour old nitrate plants, and mixed with MCC. The levels of NADH-nitrate reductase reconstituted (Table 20, experiments 4 and 5) were, however, lower than those obtained in the initial experiments where apoprotein had been isolated by sucrose density gradient centrifugation.

Experiments 1-5 all used apoprotein derived from 90-hour old nitrate plants. However, as was shown in Chapter 2 of these Results, the levels of the smaller NADH-cytochrome c reductase species (used here as apoprotein for reconstitution of nitrate reductase) are elevated in plants older than 90 hours. An extract from 144-hour old barley plants was therefore subjected to filtration through Biogel A1.5 m and aliquots from the peak of smaller NADH-cytochrome c reductase species (see Fig. 38) used for the reconstitution of nitrate reductase activity. However, the resultant NADH-nitrate reductase activity (Table 20, experiment 6) was found to be 20% less

than that of the controls (N.B. the MCC preparation possesses residual NADH-nitrate reductase activity). The possibility of this being due to a protease in the apoprotein-containing fractions was tested in a subsequent experiment (Table 20, experiment 7) by the addition of 3% BSA to the samples for reconstitution. This was found to prevent the loss of NADH-nitrate reductase activity, providing support for the existence of a protease among the apoprotein-containing fractions, but no reconstitution of NADH-nitrate reductase activity was obtained.

DISCUSSION

Throughout the attempts to reconstitute barley nitrate reductase (Table 20) the highest activity obtained was only 66 nmoles nitrite formed/hour per ml. Hewitt, Notton and Rucklidge (1977) however, using the 3.7 S NADH-cytochrome c reductase from Mo-deficient spinach plants as apoprotein, reconstituted an activity of 3.6 nmoles nitrite formed per minute. However, as the volume to which this refers was not reported, direct comparison with the results reported here is difficult, but it would appear likely that the values reported here (Table 20) are much lower than those of Hewitt, Notton and Rucklidge (1977).

It may be concluded, therefore, that no significant reconstitution of barley nitrate reductase has been obtained, which is rather surprising in view of the apparently high levels of activity reconstituted by Hewitt, Notton and

Rucklidge (1977). This difference could be explained if the apoprotein derived from Mo-deficient spinach plants is different from that derived from Mo-sufficient barley plants such that some essential component of nitrate reductase was not present in the fractions used for apoprotein from barley plants.

Alternatively, the results may be explained if the method used for the separation of MCC from spinach nitrate reductase (Hewitt, Notton and Rucklidge, 1977) is not effective with the nitrate reductase from barley. Thus the observed lack of reconstitution could be due to lack of functional MCC. Acid-treatment is not always an effective method for the preparation of MCC as it has been reported (Garrett and Cove, 1976) that acid-treated *A. nidulans* nitrate reductase can spontaneously reassociate to form holoenzyme, as soon as the pH is restored to 7. Such a situation could possibly account for some of the results obtained with barley nitrate reductase, especially if acid-treatment somehow results in loss of NADH-dehydrogenase activity as would be the case if an iron-sulphur centre were involved. Thus the enzyme would spontaneously reassociate at pH 7 but would be catalytically inactive, whilst very little MCC was left for reconstitution with the added apoprotein.

The implications of the results obtained here and those of Hewitt, Notton and Rucklidge (1977) for the structure of higher plant nitrate reductase will be dealt with in the General Discussion.

GENERAL DISCUSSION

The work reported in this thesis has been aimed at providing a better understanding of the structure and properties of higher plant nitrate reductase. However, as described in the Introduction to this thesis, all the assimilatory nitrate reductases from fungal, algal and higher plant sources have many features in common and it would therefore seem pertinent not to view any one of these enzymes in isolation from the others. For this reason, I shall first review the information available regarding the structure of the nitrate reductases from fungal and algal sources and then relate this to the data available regarding higher plant nitrate reductase.

SECTION I - NITRATE REDUCTASE FROM *ASPERGILLUS NIDULANS*

A. nidulans nitrate reductase was first purified by Cove and Coddington (1965) who showed that the enzyme was specific for NADPH as the electron donor and required exogenous FAD for both NADPH-nitrate reductase activity and NADPH-cytochrome c reductase activity. The absorption spectrum of the purified enzyme revealed a peak at 420 nm which was taken to indicate the presence of a cytochrome. Support for this was subsequently obtained by MacDonald and Coddington (1974) who obtained an absorption spectrum, for purified *A. nidulans* nitrate reductase, which indicated the presence of either a b- or a c-type cytochrome. Conflicting results were, however, obtained by Downey (1971) who was unable to detect a cytochrome component with his purified enzyme despite

being able to detect spectral changes due to oxidation and reduction of the FAD component.

Analysis of the FAD content of *A. nidulans* nitrate reductase by Downey (1973a) indicated the presence of 1 mole of FAD per mole of enzyme. However, as previously indicated, exogenous FAD must be present for the expression of NADPH-nitrate reductase and NADPH-cytochrome c reductase activities and it would therefore seem likely that the value of 1 mole FAD/mole of enzyme is a minimal estimate. Downey (1973a) also reported the presence of 1 mole of Mo/mole of enzyme.

SDS-polyacrylamide gel electrophoresis of purified *A. nidulans* nitrate reductase (Downey and Focht, 1974) revealed the presence of only one protein-staining band which corresponded to a molecular weight of 49 000. These authors therefore proposed that the enzyme was composed of four identical subunits, each of 49 000 molecular weight, giving the observed (Downey, 1971) molecular weight of 197 000 for intact *A. nidulans* nitrate reductase.

At about the same time, a conflicting model was proposed by MacDonald, Cove and Coddington (1974). This was based on the observation that several mutants lacking nitrate reductase activity uniquely possessed a species of NADPH-cytochrome c reductase activity which had a sedimentation coefficient of 4.5 S and was likely therefore to represent either a subunit or a breakdown product of nitrate reductase. The only nitrate-reductase

negative mutant which never possesses this 4.5 S species is *cnx* E (Downey, 1973b; MacDonald, Cove and Coddington, 1974) which had been previously shown to be repairable by growth in the presence of 33 mM molybdate (Arst, MacDonald and Cove, 1970) and which therefore presumably possesses a structurally intact Mo-cofactor, lacking only the Mo molecule. MacDonald, Cove and Coddington (1974) estimated the molecular weight of the 4.5 S NADH-cytochrome c reductase species to be 100 000 and therefore proposed that *A. nidulans* nitrate reductase was a dimer of the 100 000 molecular weight species and that dimerisation was brought about by the presence of intact Mo-cofactor. It was also proposed that the 100 000 molecular weight subunit was the product of the *nia* D gene.

The molecular weight of the 4.5 S NADPH-cytochrome c reductase species was, however, not accurately determined (the method of estimation was not reported) with the result that this model is based solely on an estimated value. Evidence that this value is incorrect has been provided by Lewis (1975) who measured molecular weights by means of the gel electrophoretic methods of Rodbard and Chrambach (1971). Using NADPH-tetrazolium reductase activity as analogous to NADPH-cytochrome c reductase activity, he was able to detect bands of activity which were only present after gel electrophoresis of extracts from mutants known to possess the 4.5 S NADPH-cytochrome c reductase species. These unique bands were always heterogeneous and

corresponded to an average molecular weight of 57 400.

Lewis (1975) then made the assumption that the cytochrome component of *A. nidulans* nitrate reductase would be coded for by the same gene as that responsible for the mitochondrial cytochromes and therefore any mutation in this gene would be lethal. This would explain the absence of any cytochrome-deficient mutants with altered nitrate reductase. Thus, the cytochrome component of nitrate reductase must be present in all non-lethal mutations. Lewis (1975) therefore proposed that the 4.5 S NADPH-cytochrome c reductase species was an adduct of the *nia* D gene product and a b-type cytochrome and, further, that *A. nidulans* nitrate reductase was composed of four *nia* D gene products, four cytochromes and two Mo-cofactor molecules. Each cytochrome was assumed to have a molecular weight of 12 000 and the Mo-cofactor to have a molecular weight of 5 000 - 10 000. Thus the *nia* D gene product would have a molecular weight in the range 34 000 - 43 000.

Recent work (Downey and Steiner, 1979) has cast some doubt on some of the assumptions made by Lewis (1975). Downey and Steiner (1979) subjected purified *A. nidulans* nitrate reductase to polyacrylamide gel electrophoresis and detected four protein-staining bands, two of which possessed reduced benzyl viologen nitrate reductase activity and which both stained for non-haem iron. Neither of these two bands stained for haem iron. In contrast, of the two

other protein-staining bands on the gel, neither stained for non-haem iron but one was shown to stain for haem. The reduced versus oxidised difference spectra of this latter component showed it to be a cyt b_{557}. As no bands possessed NADPH-nitrate reductase activity, Downey and Steiner (1979) concluded (a) that the enzyme had broken down to form different associations of subunits and therefore (b) cyt b_{557} was a component of *A. nidulans* nitrate reductase, in contrast to previous results (Downey, 1971).

However, another interpretation of the data of Downey and Steiner (1979) is possible. The molecular weights for the protein-staining bands were determined from their relative mobilities at different acrylamide concentrations (Hendrick and Smith, 1968) and those for the two bands possessing reduced methyl viologen nitrate reductase activity were found to be 240 000 and 118 000. It would seem likely that this latter value represents a half-molecule of the 240 000 molecular weight protein which itself is similar to the known molecular weight of *A. nidulans* nitrate reductase. If this protein does represent intact nitrate reductase then it is clear that the cyt b_{557} is not an integral part of the enzyme. The fact that this band did not possess NADPH-nitrate reductase activity can be explained as follows. Downey and Focht (1974) have previously shown that this activity decays rapidly during polyacrylamide gel electrophoresis and Lewis (1975) reported that this activity could only be detected following electro-

phoresis if FAD was included in the gels. This is not surprising as exogenous FAD is required for the assay of NADPH-nitrate reductase in fungal extracts (Cove, 1966).

It is possible, therefore, that cyt b_{557} is not a component of *A. nidulans* nitrate reductase but is instead a component of a closely-associated protein. This possibility re-raises the question of the identity of the 4.5 S NADPH-cytochrome c reductase species observed in some nitrate reductase-negative mutants. If there is no cytochrome involved in the structure of nitrate reductase then it is unlikely that the 4.5 S species is an adduct of the *nia* D gene product and that cytochrome, as suggested by Lewis (1975).

Lewis (1975) noted some heterogeneity among the bands allegedly corresponding to 'the 4.5 S' species but he failed to determine accurate molecular weights for these. Recent results from this laboratory have confirmed that more than one NADPH-cytochrome c reductase species is present in extracts from these mutants but accurate molecular weights are not available yet (J.L. Wray, unpublished observations). It is clear, however, that there is not one discrete type of '4.5 S' NADPH-cytochrome c reductase and this argues against the type of model proposed by Lewis (1975) and slightly modified by Cove (1979) in which *A. nidulans* is composed of four identical subunits held together by Mo-cofactor. It is possible, from the analysis of NADH-cytochrome c reductase species reported in Chapter 2

of the Results, and from the very similar characteristics of the nitrate reductases from barley and *A. nidulans*, to suggest that the likely molecular weight for 'the' 4.5 S NADPH-cytochrome c reductase species is within the range 70 000 - 90 000.

Although these unique NADPH-cytochrome c reductase species are not usually detected in extracts from wild-type mycelia Lewis (1975) has shown that these are produced during storage of a wild-type extract at 4°C. Thus these species can be generated *in vitro* and are therefore unlikely to represent intact (or unmodified) gene products. The most likely explanation for these phenomena is that these species are generated as a result of proteolytic degradation of some larger gene (presumably *nia* D) product. Evidence that *A. nidulans* nitrate reductase is susceptible to attack by endogenous proteases has been presented by Garrett and Cove (1976) who showed that nitrate reductase activity could be reconstituted by co-homogenisation of *nia* D and any *cnx* mutant mycelia. However, the level of resultant activity was shown to be doubled by the presence of phenylmethyl sulphonyl fluoride (PMSF), an inhibitor of serine proteases, during co-homogenisation.

Absence of reliable data regarding these '4.5 S NADPH-cytochrome c reductase species' makes it difficult to present a structural model for the enzyme. However one which fits the available data is similar to the original model of MacDonald, Cove and Coddington (1974) in which

A. nidulans nitrate reductase is composed of a dimer of *nia* D gene products of approximately 100 000 molecular weight each which are induced to dimerise by the presence of intact Mo-cofactor. However, it is clear that the *nia* D gene product is not equivalent to 'the' 4.5 S NADPH cytochrome c reductase present in some nitrate reductase-negative mutants and it may be envisaged that the 4.5 S species is generated by proteolytic cleavage of either a mutationally-altered *nia* D gene product or, in *cnx* mutants, under conditions where this gene product is unable to dimerise.

SECTION II - NITRATE REDUCTASE FROM *NEUROSPORA CRASSA*

The structure of *N. crassa* nitrate reductase has been analysed in greater detail than has that of the nitrate reductase from *A. nidulans*. The first major characterisation of the *N. crassa* enzyme was carried out by Garrett and Nason (1967; 1969) who were able to demonstrate that a cyt b_{557} was an integral component of the enzyme and was involved in electron transfer from NADPH to nitrate. Specific reduction of the cytochrome by NADPH in the presence of FAD, followed by reoxidation of the cytochrome by the addition of nitrate, were demonstrated. The cytochrome was shown to be based on a protoporhyrin IX haem, as judged from the spectrum of its pyridine haemochromogen derivative.

A sedimentation coefficient of 8 S was determined for

the enzyme, together with a Stokes radius of 7.0 nm, which yields a calculated molecular weight of 228 000 for *N. crassa* nitrate reductase. This enzyme therefore differs from *A. nidulans* nitrate reductase in possibly two respects, (i) the *N. crassa* enzyme is significantly larger than that from *A. nidulans*, and (ii) the *N. crassa* nitrate reductase definitely contains a cytochrome as an integral component of the enzyme. It is interesting to consider the possibility that these two differences are linked, in that the extra molecular weight of the *N. crassa* enzyme is due to the presence of the cytochrome component(s). As there is only one major structural gene for each enzyme (excluding genes coding for the Mo cofactor), namely *nia* D in *A. nidulans* and *nit*-3 in *N. crassa*, it would seem likely that the *nit*-3 gene has arisen by a process of gene fusion between a *nia* D type gene and a gene coding for cyt b_{557}. As *N. crassa* is more evolutionary advanced than *A. nidulans* (E. Duncan, personal communication) this type of modification would not be unexpected. Further examples of gene fusion will be given later in this Discussion.

Recently, Pan and Nason (1978) have purified *N. crassa* nitrate reductase 5 000-fold to apparent homogeneity, and confirmed the molecular weight reported by Garrett and Nason (1969). SDS gel electrophoresis of the purified enzyme revealed two protein bands, corresponding to molecular weights of 115 000 and 130 000. However, the ratio of these two bands differed between enzyme samples and proteolytic mapping of the two bands revealed identical patterns. Further, only one N-terminal amino acid (glutamic

acid) was detected and these results prompted the authors to suggest that the 130 000 molecular weight subunit was a modified form of the 115 000 molecular weight subunit and that *N. crassa* nitrate reductase consists of two similar (if not identical) subunits each with a molecular weight of 115 000. The same authors also showed the presence of 0.89 moles of Mo and 1.54 moles of haem-iron per mole of enzyme which they took to represent 1 mole of Mo and 2 moles of cytochrome b_{557} per mole of enzyme. The FAD content was not determined due to its ease of dissociation and was assumed to be 1 FAD per subunit. Based on this data it was proposed (Pan and Nason, 1978) that *N. crassa* nitrate reductase was a dimer of two 115 000 molecular weight subunits, each possessing FAD and cytochrome b_{557}, linked together by one Mo-cofactor of about 1 000 molecular weight (Nason *et al.*, 1974).

Genetic and biochemical analysis of nitrate reductase negative mutants from *N. crassa* reveals more differences from the results obtained with *A. nidulans*. Whereas synthesis of the Mo-cofactor, essential for both nitrate reductase and xanthine dehydrogenase activities, requires several (*cnx*) genes in *A. nidulans*, only one such gene (*nit*-1) has been demonstrated in *N. crassa* (Sorger, 1964). The vast majority of *cnx* mutants of *A. nidulans* are constitutive for NADPH-cytochrome c reductase activity which is normally induced by nitrate in wild-type mycelia (Pateman *et al.*, 1964; Pateman, Rever and Cove, 1967) and this altered regulation has prompted the suggestion that intact nitrate

reductase is intimately involved in its own regulation (Cove and Pateman, 1969).

In *N. crassa*, however, mutation in the *nit*-1 locus (equivalent to the *cnx* loci) does not alter the nitrate-inducibility of NADPH-cytochrome c reductase activity (Sorger, 1964; Nason *et al.*, 1970) and therefore intact nitrate reductase can not be involved in its own regulation in *N. crassa*. The NADPH-cytochrome c reductase activity of both *cnx* and *nit*-1 mutants is reported (MacDonald, Cove and Coddington, 1974; Nason *et al.*, 1970) to have a sedimentation coefficient of 4.5 S but the accuracy of this value with respect to *A. nidulans* has recently been questioned (J.L. Wray, unpublished observations). The protein responsible for the 4.5 S NADPH-cytochrome c reductase activity of *nit*-1 mutant mycelia has recently been purified to near homogeneity and shown to have a molecular weight of about 115 000 (unpublished results of Horner, reported by Pan and Nason, 1978). If this value proves to be correct then it is clear that the 4.5 S species in *nit*-1 extracts is an intact subunit of *N. crassa* nitrate reductase and therefore corresponds to a half-molecule. This would be analogous to the situation found in *A. nidulans* (Lewis and Scazzochio, 1977) where absence of the Mo-cofactor (due to mutation in any of the *cnx* genes) results in the appearance of half-molecules of xanthine dehydrogenase. Previous estimates of the molecular weight of the 4.5 S NADPH-cytochrome c reductase species from *N. crassa* are 130 000 (Ketchum and Downey, 1975) and 84 000 (Coddington, 1976), neither

of which are compatible with the above explanation of the structure of the 4.5 S species.

Mutations in the *nit*-3 gene (the structural gene for nitrate reductase) in *N. crassa* frequently result in the production of a protein which possesses reduced methyl viologen-nitrate reductase activity but not NADPH nitrate- or NADPH-cytochrome c reductase activities, and which has a sedimentation coefficient of 6.8 S (Nason *et al.*, 1970; Antoine, 1974; Coddington, 1976). Inducible and constitutive *nit*-3 mutants exist, which again argues against intact nitrate reductase being intimately involved in its own regulation in *N. crassa*. Mutations in the equivalent gene in *A. nidulans* (*nia* D) also sometimes result in production of a protein possessing reduced methyl viologen-nitrate reductase activity but in this case the sedimentation coefficient is always the same as that of wild-type enzyme (7.8 S) (Downey, 1973a; MacDonald, Cove and Coddington, 1974).

The 6.8 S reduced methyl viologen-nitrate reductase from *N. crassa* mutant *nit*-3 has been characterised by Antoine (1974) and shown to have a molecular weight of 160 000. The protein also catalysed FMNH-nitrate reductase activity and contained cyt b_{557} with an identical absorption spectrum to that obtained (Garrett and Nason, 1967) for the NADPH-nitrate reductase from wild-type *N. crassa*. Antoine (1974) used these results to support the earlier conclusions of Sorger (1965, 1966) and Sorger and Giles

(1965) that *N. crassa* nitrate reductase was composed of two different types of subunit, one responsible for NADPH-cytochrome c reductase activity and the other responsible for FMNH- and reduced methyl viologen-nitrate reductase activities. This conclusion is not consistent with the results presented by Pan and Nason (1978) which indicated that *N. crassa* nitrate reductase is a dimer of two identical subunits, and these authors suggested that a more likely explanation is that the 6.8 S protein arises by proteolytic cleavage of an aberrant subunit, possibly resulting in the loss of the FAD-containing region.

SECTION III - NITRATE REDUCTASE FROM *RHODOTORULA GLUTINIS*

Rhodotorula glutinis is a false yeast of the Fungi Imperfecti and its nitrate reductase has recently been characterised by Guerrero and Gutierrez (1977) and shown to be very similar to *N. crassa* nitrate reductase. The sedimentation coefficient of 7.9 S and Stokes radius of 7.05 nm yield a calculated molecular weight of 230 000 and cytochrome b_{557} was shown to be a component of the enzyme. Polyacrylamide gel electrophoresis of the purified enzyme revealed two protein-staining bands one of which contained about 90% of the total protein and stained for the presence of haem. Both bands exhibited both NADH-dehydrogenase activity and reduced methyl viologen-nitrate reductase activity. The amount of protein associated with the fainter band was shown to increase upon storage of the enzyme and the absence of interaction with the haem stain

was attributed to the very small amount of protein present in the band.

The level of cytochrome b_{557} present in *R. glutinis* nitrate reductase was determined by comparison with that in *Chlorella vulgaris* nitrate reductase and shown to be 0.7 of the amount present in the algal enzyme. However, they interpreted the results of Solomonson *et al.* (1975) to indicate that *C. vulgaris* nitrate reductase (see next section) contained only 2 haem molecules per mole of enzyme from which they concluded that *R. glutinis* nitrate reductase possessed only one haem per mole of enzyme. The value obtained by Solomonson *et al.* (1975) was 2.34 moles of haem per mole of nitrate reductase which could also be interpreted as indicating that three moles of haem are present in *C. vulgaris* nitrate reductase which is consistent with the suggestion (Solomonson *et al.*, 1975) that *C. vulgaris* nitrate reductase is comprised of three identical subunits. The difference in values obtained by Guerrero and Gutierrez (1977) would then be explained solely by the difference in molecular weights between the two enzymes (356 000 for *C. vulgaris* and 230 000 for *R. glutinis*) and would suggest that the *R. glutinis* nitrate reductase contains two moles of haem per mole of enzyme.

SDS-gel electrophoresis of the purified enzyme revealed the presence of one major and one minor band possessing molecular weights of 118 000 and 115 000 respectively. It would therefore appear likely that *R. glutinis*

nitrate reductase is composed of two identical subunits, each possessing a cyt b_{557} and being loosely associated with FAD (exogenous FAD was required for maximal NADPH-cytochrome c reductase and NADPH-nitrate reductase activities). Guerrero and Gutierrez (1977) suggested, however, that the subunits were the same size but were not identical due to their interpretation that only one haem was present in the nitrate reductase molecule. This suggestion would require the existence of two separate structural genes for *R. glutinis* nitrate reductase, one for each subunit. No genetic analysis of *R. glutinis* is available and therefore this suggestion cannot be tested.

SECTION IV - THE NITRATE REDUCTASES FROM *CHLORELLA* AND *CHLAMYDOMONAS*

Chlorella vulgaris, nitrate reductase has been extensively examined by Solomonson *et al.* (1975) who showed it to be much larger than the nitrate reductases found in fungi. A sedimentation coefficient of 9.7 S and a Stokes radius of 8.9 nm were determined, yielding a calculated molecular weight of 356 000. SDS polyacrylamide gel electrophoresis of the purified enzyme yielded only one protein-staining band, corresponding to a molecular weight of 100 000.

C. vulgaris nitrate reductase also differs from the fungal nitrate reductases in that exogenous FAD is not essential for the assay of NADH-nitrate reductase and NADH-

cytochrome c reductase activities (although it does stimulate these activities in extracts from *C. fusca*, Solomonson and Vennesland, 1972) implying that the FAD component of *C. vulgaris* nitrate reductase is more tightly associated with the enzyme although it is not covalently linked (Solomonson, 1979). Solomonson *et al.* (1975) were therefore able to undertake a quantitative analysis of FAD and determined that 2.35 moles were present per mole of enzyme. An identical amount of haem was detected in the enzyme but only 1.92 moles of Mo were found per mole of enzyme.

From this data, the authors concluded that *C. vulgaris* nitrate reductase was probably composed of three subunits of equal size but could not conclude that these were identical or draw any conclusions about the arrangement of the prosthetic groups on the subunits.

Very recently, the structure of *C. vulgaris* nitrate reductase has been re-evaluated (Ramadoss and Giri, 1979). They purified the enzyme by the affinity chromatography method of Solomonson (1975) and determined its molecular weight by analytical ultracentrifugation to be 280 000 ± 10 000. The subunit molecular weight was similarly determined in the presence of 6 M guanidine hydrochloride and found to be 90 000 ± 5 000 indicating that *C. vulgaris* nitrate reductase is almost certainly composed of three subunits of the same size. The authors also gave the impression that the results of quantitative analysis of the FAD, haem and Mo components of the enzyme

were consistent with each subunit possessing all three prosthetic groups. It would therefore appear that *C. vulgaris* nitrate reductase is composed of three identical subunits whereas the nitrate reductases from fungal sources appear to be composed of two identical subunits.

Although no genetic analysis of nitrate reductase has been undertaken with *Chlorella*, another green alga *Chlamydomonas reinhardii* has been analysed by two separate groups of workers. The first group (Sosa, Ortega and Barea, 1978) obtained mutants by exposure of the cells to NTG (N-methyl-N'-nitro-N-nitrosoguanidine) and these were then selected either by their ability to grow on chlorate-containing media (for discussion, see Cove, 1976a, 1976b) or by replica-plating the cells on nitrate-containing and ammonium-containing media.

Four classes of mutants were detected, the first of which showed appreciable diaphorase activity but lacked all nitrate reductase activities and would therefore appear to be analogous to the *cnx* mutants of *A. nidulans* and the *nit*-1 mutants of *N. crassa*. The sedimentation coefficient of the NADH-cytochrome c reductase activity found in these mutants was 3.5 S. The second class of mutants had no diaphorase activity but retained high levels of FMNH-nitrate reductase activity which corresponded to a sedimentation coefficient of 8.3 S which is considerably less than the value of 10 S obtained for wild-type NADH-nitrate reductase. This class of mutants is,

therefore, probably analogous to the *nit*-3 mutants of *N. crassa* but not to the *nia* D mutants of *A. nidulans* where this activity is only expressed by a protein of the same size as wild-type enzyme. The third class of mutants expressed none of the activities associated with wild-type nitrate reductase and were therefore likely to have undergone mutation in a regulatory gene for nitrate reductase. In contrast, the fourth group had normal *in vitro* levels of the nitrate reductase activities but could not grow on nitrate, indicating that nitrate uptake may have been affected.

Broadly similar results were obtained by the other group of workers (Nichols and Syrett, 1978; Nichols, Shehata and Syrett, 1978) who obtained their mutants by exposure of *Chlamydomonas reinhardii* cells to ultraviolet radiation and selected for them by growth in the presence of chlorate. One class of mutants was shown to be unable to grow on nitrate or hypoxanthine as sole nitrogen source and is therefore equivalent to the *cnx* mutants of *A. nidulans* and *nit*-1 mutants of *N. crassa*. This mutant was not analysed biochemically. Another class of mutants was shown to lack NADH-cytochrome c reductase activity but to retain reduced methyl viologen-nitrate reductase activity associated with a protein of lower molecular weight than wild-type enzyme. The third class of mutant isolated by these authors lacked all the enzymic activities associated with nitrate reductase and was presumably, therefore, a regulatory mutant.

Thus, genetic analysis of algal nitrate reductases produces results almost identical to those obtained with the fungus *N. crassa* which has been shown (Pan and Nason, 1978) to consist of two identical subunits. This supports the conclusion of Ramadoss and Giri (1979) that algal nitrate reductase is composed of three identical subunits, each possessing FAD, cytochrome b_{557} and Mo.

SECTION V - NITRATE REDUCTASE FROM HIGHER PLANTS

Other than the work reported in this thesis, the only source of information regarding the physical properties of a higher plant nitrate reductase is Hewitt's group based at Long Ashton Research Station. Notton, Fido and Hewitt (1977) reported that spinach nitrate reductase had a calculated molecular weight of 197 000, based on the experimentally determined values for sedimentation coefficient (8.1 S) and Stokes radius (6.0 nm). The same authors also identified cytochrome b_{557} as a component of the purified enzyme although the precise absorption maxima were subsequently slightly amended (Hewitt, Notton and Garner, 1979). The midpoint potential of the cytochrome b_{557} component of spinach nitrate reductase was shown to be -60 mV (Fido *et al.*, 1979) and FAD has also been shown to be a component of the enzyme (Notton and Hewitt, 1979).

SDS gel electrophoresis of the purified spinach nitrate reductase (Notton and Hewitt, 1979) revealed the presence of three major protein-staining bands corresponding

to molecular weights of 37 000, 74 000 and 120 000. Based on these results and the fact that the molecular weight for spinach nitrate reductase calculated from only its sedimentation coefficient of 8.1 S is 152 000, it has recently been proposed (Notton, Fido and Hewitt, 1979) that spinach nitrate reductase is a tetramer with a monomeric molecular weight of 37 000. This model is very difficult to reconcile with the authors own published data. Firstly, they (Notton, Icke and Hewitt, 1976) have shown that spinach nitrate reductase has an axial ratio of 10:1 which indicates that a molecular weight determination based on only one parameter is likely to be highly erroneous. The effect of this asymmetry can be clearly seen in Chapter 2 of this thesis where barley nitrate reductase is eluted from Sephadex G200 before catalase and yet sediments at a much slower rate than catalase during sucrose density gradient centrifugation. Clearly, the calculated molecular weight of 197 000 for spinach nitrate reductase is likely to be a much better estimate.

If this value is taken, and the monomeric molecular weight is still assumed to be 37 000 then it is clear that at least five such subunits would have to be present to account for the molecular weight. Notton and Hewitt (1979) also suggest that both FAD and cytochrome b_{557} are essential for the expression of NADH-cytochrome c reductase activity which they imply to be carried by the individual subunit (and considered by them to be equivalent to the 3.7 S NADH-cytochrome c reductase species -

Notton, Icke and Hewitt, 1976). This model therefore indicates that if all the subunits are identical then all will contain both FAD and cytochrome b_{557}, with the result that intact spinach nitrate reductase would contain four or five moles of both FAD and cytochrome b_{557} per mole of enzyme. There is no evidence for such a situation in any nitrate reductase. Alternatively, the subunits may all be the same size but may be of more than one type, e.g. some containing FAD and cytochrome b_{557} (in order to be able to catalyse NADH-cytochrome c reductase activity) and others containing neither component. This situation would require the existence of more than one major structural gene for higher plant nitrate reductase. Although this possibility cannot be directly examined at present it can be said that this situation, if it occurred, would be unique among the assimilatory nitrate reductases (compare with previous sections of this Discussion). It may therefore be concluded that the data presented by Hewitt's group does not allow any firm conclusions to be drawn about the structure of higher plant nitrate reductase.

The only other comprehensive set of data available regarding a higher plant nitrate reductase is that which has been presented in this thesis. Barley nitrate reductase has been shown (in Chapter 2) to have a calculated molecular weight of 203 000, based on its experimentally determined sedimentation coefficient of 7.7 S and Stokes radius of 6.4 nm. As previously indicated in this Discussion, the data available on the assimilatory nitrate reductases from

fungal and algal sources can be interpreted to indicate that all these enzymes exist either as a dimer or a trimer of a large, complex subunit, plus a low molecular weight Mo-cofactor. For the purposes of this Discussion, I will make the (apparently) logical assumption that barley nitrate reductase is no exception to this trend and is composed of two identical subunits of approximately 100 000 molecular weight each. The data presented in this thesis will then be examined in order to find support for, or against, this hypothesis.

As described in Chapter 2 of the Results, there are three major NADH-cytochrome c reductase species which can be derived from barley nitrate reductase. These have calculated molecular weights of 40 000, 61 000 and 163 000 and this largest species is also capable of catalysing NADH-nitrate reductase activity. The identity of these species is crucial for any model of the structure of higher plant nitrate reductase. If these species represent intact subunits and associations of subunits then it is clear that the proposed dimeric structure of the enzyme is not correct. The consequences of these species representing intact subunits and subunit associations have been examined in Chapter 2 of these Results and elsewhere (Wray, Small and Brown, 1979). I would like, however, to suggest that instead of representing intact structures, these species are more likely to represent catalytically active proteolytic fragments of barley nitrate reductase.

As described in Chapter 2 of the Results, the levels of those NADH-cytochrome c reductase species shown to be derived from nitrate reductase were markedly elevated in extracts from 120-hour and 144-hour old nitrate plants, while the levels of nitrate reductase itself were progressively lower from older plants. This age-dependent *in vitro* instability of nitrate reductase is well documented (Schrader, Cataldo and Peterson, 1974) and has been shown to be prevented by extraction in the presence of 3% (w/v) BSA or casein. Either of these compounds could protect nitrate reductase from proteolysis by acting as alternate protease substrates. Further, it has been recently shown in this laboratory (J. Brown, unpublished observations) that extraction of 144-hour old shoots from nitrate plants in the presence of 3% (w/v) BSA not only stabilises nitrate reductase activity, but also prevents the accumulation of the NADH-cytochrome c reductase species shown here to be derived from nitrate reductase. The existence of highly reactive proteases in extracts from plants older than 90 hours is also supported by the results presented in Chapter 8 of the Results where it was shown that addition of 3% (w/v) BSA prevented losses of nitrate reductase activity during attempts to reconstitute nitrate reductase.

Since the same types of NADH-cytochrome c reductase species are always found in extracts from older barley plants it follows that they must be produced by a specific mechanism and not just by random cleavage. Thus, the

40 000 molecular weight NADH-cytochrome c reductase species would be released as a result of cleavage of barley nitrate reductase at a specific site, which would also result in the appearance of the 163 000 molecular weight species. This larger species would retain NADH-cytochrome c reductase activity by virtue of possessing one intact 100 000 molecular weight subunit which would also allow transfer of electrons from NADH to the Mo site with the result that this protein can still catalyse NADH-nitrate reductase activity (Fig. 35). Small amounts of this species would not be detected in extracts from 90-hour old plants due to the very high levels of intact nitrate reductase. The 163 000 molecular weight species has been shown (Fig. 39) to give rise to the 40 000 molecular weight NADH-cytochrome c reductase species. It would seem likely that this process would also give rise to a 120 000 molecular weight protein capable of catalysing reduced methyl viologen-nitrate reductase activity, although this possibility was not tested.

The occurrence of small amounts of a 61 000 molecular weight NADH-cytochrome c reductase species during break-down of barley nitrate reductase can have two possible explanations. The first is that following release of the 40 000 molecular weight component the resulting 163 000 molecular weight molecule dissociates to release an intact 100 000 molecular weight subunit and a 61 000 molecular weight fragment which is somehow capable of catalysing a very low level of NADH-cytochrome c reductase activity.

Alternatively, a small proportion of the total nitrate reductase may be cleaved by a separate mechanism which results in the appearance of a 61 000 molecular weight NADH-cytochrome c reductase species rather than a 40 000 molecular weight species. This cleavage could also occur in the intact 100 000 molecular weight subunit following preliminary cleavage of the 40 000 molecular weight NADH-cytochrome c reductase from nitrate reductase.

Some support for the proposed dimeric structure of barley nitrate reductase was also obtained in Chapter 1 of the Results where purified enzyme was subjected to SDS polyacrylamide gel electrophoresis. Two major bands were detected corresponding to molecular weights of 205 000 and 105 000 and these may therefore represent intact nitrate reductase and the subunit of nitrate reductase, respectively. The other major bands, corresponding to molecular weights of 77 000 and 56 000, cannot be explained in terms of this model and may represent impurities in the enzyme sample. If this model is correct, then the 40 000 molecular weight NADH-cytochrome c reductase species is likely to represent an FAD-containing domain of the 100 000 molecular weight subunit. The fact that this species is always observed even in extracts from 90-hour old nitrate plants may therefore indicate that the proposed specific protease is always present and hence the observed breakdown pattern may reflect the *in vivo* mechanism of turnover of nitrate reductase. Further support for this will be presented later in this Discussion.

SECTION VI - SIGNIFICANCE OF DOMAIN STRUCTURES AND COMPARISON WITH OTHER ENZYME SYSTEMS

A domain is defined as an independently folded, functionally intact region of a polypeptide chain and this concept was first brought to prominance by Edelman *et al.* (1969) through their analysis of γ G-immunoglobulin. A similar proposal that proteins may be composed of 'poly globular' structures was proposed at about the same time by Goldberg (1969) and this concept has now been confirmed for several proteins.

Limited proteolysis of methionyl-tRNA synthetase by trypsin was shown (Cassio and Waller, 1971) to cleave each subunit into two distinct parts, one of which retained the full catalytic activity. The authors proposed that gene fusion had occurred to account for the structure of the intact subunit. Similar conclusions were reached about the threonine-sensitive homoserine dehydrogenase and aspartokinase activities of *E. coli* K12 (Veron, Falcoz-Kelly and Cohen, 1972). Mild proteolysis of this enzyme resulted in two fragments each of which was capable of catalysing one of the enzymic activities.

Flavocytochrome b_2 from baker's yeast was originally thought (Mevel-Ninio, 1972) to be composed of two types of subunit, one type containing the cytochrome component of the enzyme and the other type containing the flavin component. Subsequent work (Naslin, Spyridakis and Labeyrie, 1973) however, showed the enzyme to be polyglobular in

structure, with the globules held together by flexible and loosely structured regions which were hypersensitive to attack by proteases. This was confirmed by Jacq and Lederer (1974) who proposed a bi-globular structure, with the FMN and cytochrome components located on separately folded regions of the polypeptide chain. Gene fusion was again proposed to account for the observed subunit structure (Naslin, Spyridakis and Labeyrie, 1973).

The respiratory nitrate reductase from *E. coli* has also been shown (De Moss, 1977) to undergo limited proteolysis in the presence of trypsin. As a result of trypsin-treatment, the heat-released enzyme was converted from an associating-dissociating system to a non-associating monomer which retained full enzymic activity.

One of the most interesting proteins to have been shown to be composed of independent domains is rat liver sulphite oxidase. Like nitrate reductase, this enzyme is a molybdohaemoprotein and it catalyses the terminal reaction in the oxidative degradation of sulphur-containing amino acids. Trypsin cleavage of this enzyme (Johnson and Rajagopalan, 1977) results in the release of two major fragments, the smaller of which (molecular weight 9 500) contains the cytochrome component of the enzyme. The larger fragment is released as a dimer (molecular weight 100 000) which contains all the Mo of sulphite oxidase. All the epr prpperties of the Mo centre of native sulphite oxidase are retained in the Mo fragment. The authors concluded that the Mo and cytochrome components of sulphite

oxidase were contained in distinct domains which are covalently linked by means of an exposed hinge region. The length of this hinge region was later shown (Southerland *et al.*, 1978) to be about 30 residues long and the circular dichroic (CD) spectra of native sulphite oxidase was shown to represent the summation of the CD spectra of the isolated domains, indicating that proteolysis does not result in significant structural changes. It was also shown (Southerland and Rajagopalan, 1978) that the reduced form of the enzyme was less susceptible to proteolytic attack due to a change in the interdomain interaction such that the hinge region cleavage sites become less accessible.

Thus we have a protein (sulphite oxidase) which contains both Mo and a cytochrome, which has the same absorption spectrum as that from several assimilatory nitrate reductases (see the Introduction to this thesis), with each component contained in an independently-folded structural domain. Further, yeast flavocytochrome b_2 contains both flavin and cytochrome components contained in separate domains. As most of the assimilatory nitrate reductases have been shown to contain Mo, cytochrome and flavin components it would therefore seem likely that these are also contained in separate functional domains in nitrate reductase. Further, as it has been shown for each of the enzymes described in this section that the interdomain hinge regions are hypersensitive to proteolytic attack, this proposed structure for nitrate reductase

also accounts for the breakdown products derived from it. Thus the 40 000 molecular weight NADH-cytochrome c reductase species would represent an FAD-containing domain of barley nitrate reductase, whilst the 61 000 molecular weight species would represent an adduct of a flavin-containing domain and a cytochrome-containing domain. The relatively low levels of the 61 000 molecular weight species may indicate that the hinge region linking the cytochrome and Mo domains is much less accessible than is the hinge region between the flavin and cytochrome domains.

Thus, this model predicts that the 40 000 molecular weight NADH-cytochrome c reductase species does not contain a cytochrome component (see the Discussion in Chapter 3 of the Results). This can, of course be put to experimental test. Full details of the model proposed for barley nitrate reductase are presented in Fig. 58.

SECTION VII - NITRATE REDUCTASE INACTIVATING ENZYMES

Wallace (1974, 1975) has isolated a nitrate reductase inactivating enzyme from maize roots. This protein apparently attacked the NADH-cytochrome c reductase-catalysing moiety of nitrate reductase, since this activity is inactivated to a greater extent than FMNH- and reduced methyl viologen-nitrate reductase activites. The activity of the inhibitor was prevented by inclusion of phenylmethylsulphonyl fluoride (PMSF) indicating that the

FIG. 58

A Model for the Structure of Higher Plant Nitrate Reductase and for the Structures of the NADH-cytochrome c Reductase Species Derived from Nitrate Reductase

A Proposed structure of the subunit of higher plant nitrate reductase.

I - the 40 000 molecular weight FAD-containing domain

II - the 20 000 molecular weight haem-containing domain

III - the 40 000 molecular weight MCC-binding domain

... denotes hinge regions.

B Proposed structure of higher plant nitrate reductase. It is envisaged that MCC (molecular weight approx. 1 500) induces the individual subunits to dimerise. Thus intact nitrate reductase would contain two flavins two haems and one MCC.

C Proposed structure of the 40 000 molecular weight NADH-cytochrome c reductase species (= the FAD-containing domain).

D Proposed structure of the 61 000 molecular weight NADH-cytochrome c reductase species (= adduct of FAD-containing domain and haem-containing domain).

E Proposed structure of the 163 000 molecular weight nitrate reductase/NADH-cytochrome c reductase species (= intact nitrate reductase lacking one FAD-containing domain).

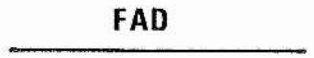
A


FAD
HAEM
I
II
III
40 000
20 000
40 000

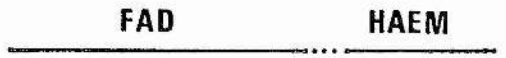
B


FAD
HAEM
FAD
HAEM
MCC

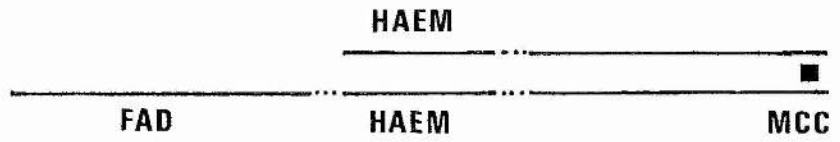
C


FAD

D


FAD
HAEM

E


HAEM
FAD
HAEM
MCC

inhibitor is a serine protease. The inhibitor was claimed (Wallace, 1978) to be specific for nitrate reductase but was shown to hydrolyse azo-casein, and to have a molecular weight of about 40 000.

A nitrate reductase-inactivating enzyme has also been detected in rice roots (Kadam *et al.*, 1974) and in cultured rice cells (Yamaya and Ohira, 1976, 1977, 1978). The enzyme from cultured rice cells was shown to have a molecular weight of about 200 000, to be heat labile and to inhibit all the nitrate reductase-associated activities except reduced methyl-viologen-nitrate reductase activity. The nitrate reductase-inactivating enzymes from rice cells and maize roots have been recently compared (Yamaya and Oaks, 1979) and found to be quite different with respect to molecular weight, electrophoretic mobility and ability to hydrolyse azo casein. NADH had no effect on the enzyme from maize roots, but inhibited the enzyme from rice cells confirming the previous results of Kadam *et al.* (1974).

A nitrate reductase-inhibitor has also been isolated from soy bean leaves (Jolly and Tolbert, 1978). This protein was heat-labile, had a molecular weight of about 31 000 and consisted of two identical subunits. The enzyme completely inhibited NADH-nitrate reductase activity, but its effect on both FMNH- and reduced methyl viologen-nitrate reductase activities was much less marked. NADH-cytochrome c reductase activity was not affected. This is the type of result which would be expected if the

nitrate reductase-inhibitor was the type of specific protease proposed to exist in barley. The inhibitor was unable to hydrolyse a range of synthetic polypeptides and was not inhibited by PMSF from which the authors concluded that it was not a protease. However, PMSF is very unstable in solution (J.R. Coggins, personal communication) and so may not fully inhibit serine protease unless it is freshly prepared. Alternatively, the inhibitor may not be a serine protease. Also, if the inhibitor were a protease specific for nitrate reductase it would not be expected to hydrolyse synthetic polypeptides. The authors also found evidence for a specific activator of nitrate reductase in soybean extracts which supports the earlier results of Purvis and Tischler (1976) with cotton.

Very recently, several factors which affect the stability of nitrate reductase have been isolated from wheat leaves (Sherrard, Kennedy and Dalling, 1979a, b). Two factors were found to stabilise nitrate reductase *in vitro* whilst the other was found to inhibit the enzyme. This inactivating factor was heat labile and had a molecular weight of about 37 500. As with the inhibitor isolated from soybean leaves (Jolly and Tolbert, 1978), the inactivating factor from wheat leaves was most active towards NADH-nitrate reductase activity, while FMNH- and reduced methyl viologen nitrate reductase activities were much less affected. Again NADH-cytochrome c reductase activity was unaffected as would be expected if this inactivating enzyme were equivalent to the nitrate

reductase specific protease proposed to exist in barley shoots. Neither casein or a range of artificial substrates were hydrolysed by the wheat inactivating enzyme and it was not inhibited by PMSF. It was, however, inhibited by the trypsin inhibitors α-p-tosyl-L-lysine chloromethyl ketone and α-N-benzoyl-L-arginine.

Based on these results I would like to propose that the nitrate reductase-inactivating enzymes isolated from soybean leaves and wheat leaves are the same type of enzyme and that this protein is specifically capable of proteolytically attacking the proposed hinge region between the flavin and cytochrome domains of higher plant nitrate reductase. This would have the effect of abolishing NADH-nitrate reductase activity while still permitting NADH-cytochrome c reductase activity to be catalysed by the structurally-intact flavin domains.

SECTION VIII - RECONSTITUTION AND MECHANISM OF NITRATE REDUCTASE TURNOVER

If, as proposed here, the NADH-cytochrome c reductase species released from nitrate reductase result from proteolytic action and do not represent combinations of intact subunits, it is at first difficult to envisage how the Long Ashton workers (Rucklidge, Notton and Hewitt, 1976; Hewitt, Notton and Rucklidge, 1977) were able to reconstitute nitrate reductase activity *in vitro*. However, it has been previously shown that separated functional domains of an enzyme can be reconstituted into active enzyme

possessing characteristics very close to those of the normal enzyme. The pyruvate dehydrogenase multienzyme complex from *E. coli* was originally shown (Perham and Thomas, 1971) to be degraded during storage and this was attributed to the action of a protease. It was subsequently shown (Hale and Perham, 1979) that limited proteolysis with trypsin led to release of the E_1, E_2 and E_3 components which were shown to reassemble when incubated together. Ultracentrifugational analysis revealed only a very small difference between the sedimentation coefficients of the untreated and reconstituted enzyme complexes (60 S and 57.2 S respectively) indicating that only a small amount of protein was lost during trypsin treatment.

It is interesting to analyse the method of *in vitro* reconstitution used by Hewitt and co-workers. These authors reportedly obtained their Mo-cofactor (MCC) by acid-treatment of purified spinach nitrate reductase. MCC was shown to pass through a membrane filter with a nominal cut-off at 30 000 molecular weight, but in the process lost 50% of its reconstituting activity. Passage through a membrane filter with cut-off at 10 000 molecular weight led to loss of reconstituting activity, even when the filtrate and retentate were mixed. Thus MCC from spinach nitrate reductase would appear to have a molecular weight of between 10 000 and 30 000. However, if MCC were this size then its absence from nitrate reductase could be detected by sucrose density gradient centrifugation

(MacDonald and Cove, 1974; Cove, 1979). This suggests that the 'active ingredient' obtained from spinach nitrate reductase may not just be MCC. This is supported by results with MCC from *N. crassa* (see later) which show MCC to have a molecular weight of about 1 500.

Also, as source of apoprotein, the Long Ashton workers use the trailing edge of NADH-cytochrome c reductase species C which has been shown in Chapter 2 of the Results to be high in the 27 800 molecular weight NADH-cytochrome c reductase species which is not related to nitrate reductase. The only nitrate reductase-related NADH-cytochrome c reductase species likely to be present in their sample is the 3.1 S (40 000 molecular weight) species. Thus the problem of just what is contained in the MCC preparations is of great importance. If only MCC were present then *in vitro* reconstitution would represent an MCC-catalysed association of identical NADH-cytochrome c reductase - catalysing subunits.

The Long Ashton workers believe that 'the' cytochrome c reductase requires a cytochrome for its activity and would therefore possess both flavin and cytochrome components. Thus, the reconstitution they propose would require nitrate reductase to contain four haems and four flavins. This situation is not likely.

If, however, as the model presented here (Fig. 58) suggests, that no cytochrome is present in the 40 000 molecular weight NADH-cytochrome c reductase, then it

follows that reconstitution between that species and MCC would be limited by the amount of cytochrome present. This model predicts that the MCC preparation of Hewitt and co-workers must contain all the structural components of nitrate reductase except the flavin-containing domain.

Hewitt and co-workers have also reported *in vitro* reconstitution of spinach nitrate reductase by mixing MCC with a total extract from Mo-deficient plants. Some important points can be drawn from their data.

(a) As a control, they mix apoprotein (from Mo-deficient spinach) with an equal volume of extraction buffer instead of with the pH 6.2 buffer which is used for MCC. This difference in pH would be likely to affect enzyme activity.

(b) Very little attention was paid to sedimentation coefficient. They report that the types of NADH-cytochrome c reductase species found in extracts from Mo-deficient spinach plants differ from those in Mo-sufficient plants, and have almost equal amounts of light and heavy species. The species have reported sedimentation coefficients of 8.1 S, 6.9 S, 5.5 S and 3.6 S. These values and the overall profile are very similar to those reported here for 120-hour old barley plants.

(c) Please refer to either Fig. 2 in Rucklidge, Notton and Hewitt (1975) or Fig. 5 in Hewitt, Notton and

Rucklidge (1977). During reconstitution, the authors report a drop in the level of the smaller NADH-cytochrome c reductase species and an increase in the level of the larger species. However, analysis of the above-mentioned figures reveals that there was no 8.1 S NADH-cytochrome c reductase species in the Mo-deficient extract used - the major peak is associated with the 6.8 S species. Thus it may be concluded that nitrate reductase is broken down in the absence of Mo and this gives rise to the elevated NADH-cytochrome c reductase activity in the 3-4 S region as well as high amounts of a 6.8 S species. Also of note from these figures is the fact that following reconstitution, the NADH-cytochrome c reductase activity in the 3-4 S region (fractions 4-7) is obviously heterogeneous, supporting the conclusions reached in Chapter 2 of the Results.

The data presented by Hewitt and co-workers is clearly compatible with the suggestion that *in vitro* reconstitution of spinach nitrate reductase is due to the re-association of intact, functional domains.

The apparent proteolysis reported in this thesis is most likely to occur during extraction of the nitrate reductase since addition of 3% (w/v) BSA results in the 'classic' distribution of NADH-cytochrome c reductase

activities found in extracts from 90-hour old plants (J. Brown, unpublished observations). However, it is clear that analogous, if not identical, NADH-cytochrome c reductase species are present in extracts from 120-hour old barley plants as are found in extracts from Mo-deficient spinach plants. As these species are not observed in Mo-sufficient spinach plants of the same age (Notton, Icke and Hewitt, 1976) it may be concluded that these NADH-cytochrome c reductase species are likely to be generated *in vivo* as a consequence of Mo-deficiency.

Thus, in the absence of Mo, the altered nitrate reductase protein appears to be broken down. Enhanced turnover of altered proteins is well documented (Platt, Miller and Weber, 1970; Lin and Zabin, 1972; De Simone *et al.*, 1974) and it would therefore appear likely that the types of NADH-cytochrome c reductase species seen in Mo-deficient plants reflect the mechanism of *in vivo* turnover of nitrate reductase. It is therefore of major interest to see that the types of NADH-cytochrome c reductase species present in Mo-deficient spinach plants are very similar to those found in extracts from 120-hour barley plants, and provides strong evidence in favour of the suggestion made here that these species arise as a consequence of proteolytic action.

Moreover, these results also offer an explanation for the ubiquitous presence of the 40 000 and 61 000 molecular weight NADH-cytochrome c reductase species in extracts

from nitrate barley plants. The appearance of low levels of these species, even following extraction in the presence of 3% BSA, suggests either that (a) these species can be derived by non-catalytic breakdown of nitrate reductase or, more likely, (b) that proteolytic breakdown of nitrate reductase occurs even in 90-hour old nitrate plants due to normal turnover. Thus, the levels of these species present in extracts from 90-hour old nitrate plants, or in extracts from older plants prepared in the presence of 3% (w/v) BSA, reflects the extent of *in vivo* turnover of nitrate reductase.

Based on all these results it is also possible to suggest a mechanism for the *in vivo* turnover of barley nitrate reductase. The predominance of the 40 000 molecular weight NADH-cytochrome c reductase among the breakdown products suggests that the initial site of cleavage is the proposed hinge region between the flavin and cytochrome domains of the enzyme. This would be done by a nitrate reductase-specific inactivating enzyme, possibly equivalent to those recently isolated from soybean (Jolly, and Tolbert, 1978) and wheat (Sherrard, Kennedy and Dalling, 1979a, b). The resultant 163 000 molecular weight NADH-cytochrome c reductase species has not been detected in extracts from 90-hour old nitrate plants probably because its low levels of activity were masked by the very high level of intact nitrate reductase still present. These released breakdown products would then be attacked by less

specific proteases. This mechanism of degradation - attack by a specific protease followed by breakdown of the released fragments - has gained widespread acceptance following the discovery of specific proteases which attack the apo-forms of pyridoxal-phosphate dependent enzymes (Katunuma *et al.*, 1976) and other proteins (Segal, 1976; Kirschner and Bisswanger, 1976).

As indicated in the Introduction to this thesis, soybean nitrate reductase appears to be different from most other higher plant nitrate reductases in that both NADH and NADPH can act as electron donors. Wells and Hageman (1974) were unable to separate these activities but Jolly, Campbell and Tolbert (1976) managed to separate the two activities by gel filtration. The Stokes radius of the NADH-dependent protein appears to be about 6.4 nm whilst that of the NADPH-dependent protein appears to be about 5 nm (my analysis of their data). It was subsequently shown (Iranzo and Campbell, 1979) that the NADH-dependent activity was highest in extracts from 8-day old plants whilst the NADPH-dependent activity was highest in 10-day old plants.

In view of the results presented in this thesis it is tempting to suggest that the protein responsible for NADPH-nitrate reductase activity is derived from that responsible for NADH-nitrate reductase activity as a result of proteolytic cleavage. Insufficient data is available to allow speculation about the possible structure of the NADPH-dependent species. The apparent

increase in protease activity with respect to plant age reported here for barley offers an explanation for the two nitrate reductase activities of soybean reaching maximum levels at different plant ages. Thus, if the NADPH-dependent species were derived from the NADH-dependent species, its level would continue to rise for a while after the level of the NADH-dependent species began to fall. It would be interesting to determine if the NADPH-nitrate reductase species is detected if the extraction is carried out in the presence of 3% (w/v) BSA as this would show whether it is present *in vivo* or is generated solely during extraction.

SECTION IX - FACTORS AFFECTING PROTEIN BREAKDOWN *IN VIVO*

One of the major conclusions of this thesis is that higher plant nitrate reductase may be susceptible to proteolytic attack which can result in the release of intact, functional domains. It would seem pertinent, therefore, to consider the factors which are known to influence protein breakdown *in vivo*.

(a) Size

Most work done on the control of protein turnover has been done in animal cells, but the results are likely to be valid for higher plants. One of the major factors controlling protein breakdown *in vivo* is protein size (Dehlinger and Shimke, 1970; Dice, Dehlinger and Schimke, 1973) such that

larger proteins have higher turnover rates and hence short half-lives. Higher plant nitrate reductase is a large protein and this correlates well with the very short half-life reported for the spinach nitrate reductase (Notton, Hewitt and Fielding, 1972).

(b) Isoelectric Point

The work of Dice and Goldberg (1975) has shown that acidic proteins underwent more rapid rates of catabolism than neutral or basic proteins. The relationship between isoelectric point and half-life appeared to be distinct from that between protein size and half-life. Spinach nitrate reductase is reported to be an acidic enzyme with an isoelectric point at pH 5.0 (Notton and Hewitt, 1979).

(c) Amino Acid Composition

The amide content of peptides and proteins has been suggested to be an important feature in determining the half-live of a protein (Robinson, 1974; Robinson and Rudd, 1974) although other combinations of amino acids may be important (Momany, Aguanno and Larrabee, 1976). No data is available on the amino acid composition of higher plant nitrate reductase.

(d) Cofactor/Substrate Availability

Absence of required cofactors and substrates has

been shown to affect the *in vivo* stability of several proteins (Goldberg and Dice, 1974). Evidence of this with spinach nitrate reductase has been described in the previous section of this Discussion where absence of Mo appears to result in enhanced turnover of nitrate reductase. Similarly, rat liver sulphite oxidase was less susceptible to proteolysis with trypsin in the presence of NADH than in the absence of NADH (Southerland and Rajagopalan, 1978).

It may therefore be concluded that higher plant nitrate reductase fulfills many of the criteria which are important in determining susceptibility to proteolytic attack.

SECTION X - THE NATURE OF MOLYBDENUM COFACTOR

Nason *et al.* (1970) demonstrated that *N. crassa* nitrate reductase activity could be reconstituted by mixing cell-free extracts from induced *nit*-1 mycelia with extracts from induced or uninduced *nit*-2 or *nit*-3 mycelia, or with an extract from uninduced wild-type mycelia. These results were originally interpreted to indicate that *N. crassa* nitrate reductase was composed of two components, one inducible by nitrate and present in *nit*-1 mycelia, plus a constitutive component lacking in *nit*-1 mycelia.

These results were later extended (Ketchum *et al.*,

1970; Nason *et al.*, 1971; Ketchum and Swarin, 1973; Ketchum and Sevilla, 1973) to reveal that the inducible *nit*-1 component could interact with a Mo-cofactor isolated by acid-treatment at pH 2 from any Mo-containing enzyme from animal, bacterial, and higher plant sources. It was concluded from these results that the *in vitro* formation of nitrate reductase was due to interaction of a nitrate-inducible moiety specified by *nit*-1 and a Mo-containing cofactor (MCC) found in enzymes from diverse phylogenetic sources. Lee *et al.* (1973) subsequently demonstrated incorporation of ^{99}Mo into nitrate reductase during *in vitro* formation of nitrate reductase from an extract from induced *nit*-1 mycelia and an extract from uninduced wild-type mycelia grown in the presence of ^{99}Mo.

Neither molybdate, either free or complexed with cysteine or glutathione (Lee *et al.*, 1974a) or any of several Mo-amino acid complexes (Nason *et al.*, 1970) were effective sources of MCC. However, inclusion of 10 mM molybdate during *in vitro* reconstitution resulted in significant enhancement of nitrate reductase activity (Lee *et al.*, 1974a). Vanadium (V) and Tungsten (W) which are both analogues of Mo, could be incorporated during *in vitro* reconstitution but the resultant enzyme was inactive (Lee *et al.*, 1974b).

The Mo-cofactor from *N. crassa* has been further studied recently (Lee, 1978) and shown to have a molecular weight of between 700 and 1 500 and to require EDTA, mercapto-

ethanol and 10 mM molybdate for maximal activity. Optimum conditions for reconstitution of nitrate reductase activity were established to be pH 7.4 in the presence of 0.01 M phosphate buffer. Higher buffer concentrations were inhibitory. None of these conditions is met by the method for MCC preparation used by Hewitt, Notton and Rucklidge (1977) and which was used in the work reported in this thesis. No data is available on the Mo-cofactor from *A. nidulans* but Garrett and Cove (1976) have indicated that although it is not induced by nitrate, its synthesis may be repressed by ammonium.

Recently, methods have been devised for the isolation of Mo-cofactors and that from nitrogenase has been shown (Shah and Brill, 1977) to be an iron- Mo-protein (FeMoCo). Contrary to previous results (Nason *et al.*, 1971), this Mo-cofactor is not capable of reconstituting nitrate reductase activity when mixed with an extract from induced *nit*-1 mycelia (Pienkos, Shah and Brill, 1977). A different Mo-cofactor, which did not contain iron (MoCo) was isolated by these authors from xanthine oxidase and this Mo-cofactor was capable of reconstituting nitrate reductase activity when mixed with an extract from induced *nit*-1 mycelia. The results of Nason *et al.* (1971) were explained (Pienkos, Shah and Brill, 1977) by the presence of both MoCo and FeMoCo in impure preparations of nitrogenase.

Work with another molybdoenzyme, rat liver sulphite oxidase (Jones, Johnson and Rajagopalan, 1977; Johnson,

Jones and Rajagopalan, 1977) has shown that the W-substituted enzyme can be reconstituted by addition of molybdate under very stringent experimental conditions. The mechanism of reconstitution was shown to involve loss of W from demolybdosulphite oxidase followed by replacement with Mo to form active enzyme.

Demolybdosulphite oxidase could also be reconstituted by the addition of a Mo-cofactor isolated from rat liver, *E. coli*, *N. crassa* and human tissue, but was only achieved by demolybdosulphite oxidase molecules which did not contain W. The Mo-cofactor from rat liver was found to be located on the mitochondrial outer membrane while those from the other tissue sources were either in a particulate cell fraction or associated with a macromolecule. This supports the original observation that *N. crassa* Mo-cofactor was particulate, although not of mitochondrial origin (Nason *et al.*, 1970).

The membrane-bound cofactor was stable for several days at 4° (Johnson, Jones and Rajagopalan, 1977) but activity was lost after repeated freezing and thawing, possibly due to disruption of the membrane. Optimum conditions for reconstitution were again found to be pH 7.4 and 0.01 M phosphate buffer, casting further doubt upon the method used in this thesis, and the results obtained by Hewitt and co-workers.

The carrier protein associated with the Mo-cofactor in *E. coli* was shown (Johnson, Jones and Rajagopalan, 1977)

to have a molecular weight of about 45 000 and they proposed that its function would be to provide a stabilising environment for the low molecular weight Mo-cofactor. Similar results have been obtained by Amy and Rajagopalan (1979) who reported that the Mo-cofactor could be easily released from its carrier protein by dialysis, gel filtration or ultrafiltration through a membrane of nominal 2 000 molecular weight exclusion limit. Dissociated cofactor was very sensitive to oxygen but was relatively stable when complexed.

The requirement for a stabilising protein for Mo-cofactor in *E. coli* and other tissues argues that the multiple *cnx* alleles found in *A. nidulans*, which are all required for the production of active Mo-cofactor, may be involved in the synthesis of a specific binding protein to stabilise the Mo-cofactor. As yet, however, no data is available to support this suggestion. The available data also suggest that Mo-cofactors may be products of molybdoproteins rather their natural biosynthetic precursors (Scott, Sperl and De Moss, 1979).

SECTION XI - GENETIC EVOLUTION OF THE NITRATE REDUCTASES

It has been recently discovered (see Crick, 1979) that many genes from eukaryotic sources contain inserts which do not code for protein. Transcription of the complete gene, including inserts, results in the production of heterogeneous nuclear RNA which is processed within

the nucleus to remove all the inserts in order to produce true messenger RNA.

It has been suggested (Lewin, 1979) that rather than considering the inserts as being mobile elements intruding themselves into previously intact genes, it is worthwhile to consider the genes themselves as mobile entities. Thus a 'minigene' which codes for a length of polypeptide which itself constitutes a functional area, or domain, could readily be combined with another 'minigene' to form a completely new protein. This is obviously an advantage to evolution as new genes can be formed from prefabricated sections rather than waiting for evolution to run its course.

Evidence for this suggestion is minimal as yet but for the proteins so far analysed (Immunoglobulin G heavy chain and haemoglobin) the number of inserts in the gene is one less than the number of functional domains. If this theory turns out to be a general phenomenon then it is possible to see how complex proteins like nitrate reductase, sulphite oxidase, etc. may have evolved. If the suggestions made in this thesis are correct then each of these complex proteins is likely to be composed of subunits which comprise several distinct domains, each of which would be coded for by a separate mini-gene. This may be the best explanation of the observation, reported in the Introduction to this thesis, that the cytochrome components of *N. crassa*, *Chlorella* and spinach nitrate

reductases, as well as rat liver sulphite oxidase, all possess identical absorption maxima and are therefore likely to represent very similar, if not identical, structural components.

SECTION XII - CONCLUSIONS AND THOUGHTS ON FUTURE WORK

The results presented in this thesis provide the basis for a better understanding of higher plant nitrate reductase. Barley nitrate reductase is only the second higher plant nitrate reductase to have been characterised accurately with respect to molecular weight and much of the other data presented here has no parallel in the literature. Nevertheless, much has been learned.

The apparent proteolytic breakdown of barley nitrate reductase and the types of NADH-cytochrome c reductase species released from it has allowed a model to be presented for the structure of higher plant nitrate reductase (Fig. 58). This model accounts most of the data presented here and much of that reported in the literature. Thus higher plant nitrate reductase is envisaged to consist of two identical subunits, each of 100 000 molecular weight and containing both FAD and cytochrome b_{557} in separate functional domains of the same polypeptide. It is not possible at this stage to say whether the enzyme is synthesised with an intact Mo-domain, similar to sulphite oxidase, or whether a Mo-cofactor

is added subsequent to subunit formation. I personally favour the latter possibility and consider that there is an 'MCC-binding domain' on the subunit polypeptide. MCC could thus be considered to cause dimerisation of the subunit polypeptides to form functional nitrate reductase.

Following directly from this model, a mechanism for the *in vivo* turnover of nitrate reductase has been proposed which involves an initial cleavage by a nitrate reductase-specific protease which would have properties identical to those exhibited by the nitrate reductase-inhibitors recently isolated from soybean (Jolly and Tolbert, 1978) and wheat (Sherrard, Kennedy and Dalling, 1979a, b). Finally, the proposed structure of nitrate reductase also gives an insight into its genetic evolution and its possible gene structure.

However, this is only a working hypothesis which explains most of the data reported here, and much work remains to be done before this model can be verified, or disproved. Experiments which immediately suggest themselves include:

(a) limited proteolysis of purified barley nitrate reductase to determine if the same types of NADH-cytochrome c reductase species are produced as are produced during extraction of older barley plants,

(b) purification of the proposed nitrate reductase-specific protease

(c) characterisation of proposed nitrate reductase specific protease to see if it releases a 40 000 molecular weight NADH-cytochrome c reductase species from barley nitrate reductase,

(d) analysis of the nitrate reductase-inhibitors from soybean and wheat to determine if they can release a 40 000 molecular weight NADH-cytochrome c reductase species from barley nitrate reductase

(e) characterisation of the 40 000 molecular weight NADH-cytochrome c reductase species - if the proposed model is correct then no cytochrome will be present, indicating that a cytochrome would not be required for expression of NADH-cytochrome c reductase activity

(f) purification and characterisation of the other components which are derived from nitrate reductase

(g) analysis of the Mo-cofactor - is it membrane-associated? If so, which membrane?

(h) genetic analysis - production and analysis of nitrate reductase-deficient mutants from higher plants

(i) extract and clone the gene for a higher plant nitrate reductase. Determination of the base sequence of the gene would allow the complete enzyme structure to be elucidated - including any domains.

Thus it is clear that there is a long way to go before a full understanding of the structure and control of higher plant nitrate reductase is available. However, the work described in this thesis will allow the intrepid explorer to determine which way to go.

APPENDICES

APPENDIX I

CALCULATION OF REFRACTIVE INDEX

This appendix contains a program which is suitable for a Texas TI58/59 calculator and which calculates the refractive index of a sample when applied with the reading from an Abbé type refractometer (in degrees, minutes and seconds).

Key in the following program:

Location	Key Code	Key
0		LRN
1	81	RST
2	91	R/S
3	76	2nd |b|
4	11	A
5	42	STO
6	03	03
7	65	×
8	01	1
9	00	0
10	00	0
11	95	=
12	22	INV
13	59	2nd Int
14	55	÷
15	00	0
16	93	.

Location	Key Code	Key
17	06	6
18	95	=
19	42	STO
20	04	04
21	43	RCl
22	03	03
23	22	INV
24	59	2nd Int
25	65	×
26	01	1
27	00	0
28	00	0
29	95	=
30	59	2nd Int
31	44	SUM
32	04	04
33	01	1
34	00	0
35	22	INV
36	49	2nd Prd
37	04	04
38	43	RCl
39	03	03
40	59	2nd Int
41	65	×
42	06	6
43	95	=

Location	Key Code	Key
44	42	STO
45	05	5
46	43	RCl
47	04	04
48	85	+
49	43	RCl
50	05	05
51	95	=
52	65	×
53	43	RCl
54	01	01
55	85	+
56	43	RCl
57	02	02
58	95	=
59	91	R/S
60		LRN

Enter the following numbers into the memories as shown:

0.0017412	STO 01
1.3	STO 02

Enter the reading from the refractometer in the form

2.4559 (= 2° 45' 59")

Press A

The calculator will then display the refractive index corresponding to the value entered.

APPENDIX II

GEL FILTRATION I (For use with Sephadex G200 Columns)

This program, suitable for a Texas TI58/59 calculator, converts the peak fraction number of a protein into (a) its elution volume and (b) the value of $K_d^{1/3}$ which can be plotted directly against Stokes radius according to the correlation of Porath (1963). K_d, the distribution coefficient, is defined as

$$\frac{V_e - V_o}{V_t - V_g - V_o}$$

where $V_g = V_t/B.d.$

B = bed volume per gram dry gel

d = density of gel.

Key in the following program:

Location	Key Code	Key
0		LRN
1	81	RST
2	91	R/S
3	76	2nd |b|
4	11	A
5	65	×
6	43	RCl
7	04	04
8	95	=

Location	Key Code	Key
9	91	R/S
10	76	2nd |b|
11	12	B
12	75	-
13	43	RCl
14	01	01
15	95	=
16	55	÷
17	53	(
18	43	RCl
19	02	02
20	75	-
21	43	RCl
22	03	03
23	75	-
24	43	RCl
25	01	01
26	54	)
27	95	=
28	22	INV
29	45	y^x
30	03	3
31	95	=
32	91	R/S
33		LRN

Enter void volume (V_o) of column (ml)

Press STO 01

Enter total volume (V_t) of column (ml)

Press STO 02

*Press ÷ 49.5 =

Display then shows V_g

Press STO 03

Enter volume of fractions (ml)

Press STO 04

(*This is for Sephadex G200 only.
B = 30 ml/g and
d = 1.65 g/ml. Hence B.d. = 49.5)

Enter the peak fraction number of the protein.

Press A

The calculator then displays the elution volume of the peak

Press B

The calculator then displays the value of $K_d^{1/3}$ corresponding to that peak.

APPENDIX III

GEL FILTRATION II (For gels other than Sephadex G200)

This program, suitable for a Texas TI58/59 calculator, converts the peak fraction number of a protein into (a) its corresponding elution volume and (b) the value of $(-\log K_{av})^{\frac{1}{2}}$ which can be plotted against Stokes radius using the correlation of Laurent and Killander (1964).

$$K_{av} = \frac{V_t - V_o}{V_t - V_o}$$

Key in the following program:

Location	Key Code	Key
0		LRN
1	81	RST
2	91	R/S
3	76	2nd |b|
4	11	A
5	65	×
6	43	RCl
7	03	03
8	95	=
9	91	R/S
10	76	2nd |b|
11	12	B
12	75	-

Location	Key Code	Key
13	43	RCl
14	01	01
15	95	=
16	55	÷
17	53	(
18	43	RCl
19	02	02
20	75	-
21	43	RCl
22	01	01
23	54	)
24	95	=
25	28	2nd log
26	94	+/-
27	34	$\sqrt{x}$
28	95	=
29	91	R/S
30		LRN

Enter the void volume of the column (ml)

Press STO 01

Enter the total volume of the colume (ml)

Press STO 02

Enter the fraction size (ml)

Press STO 03

Enter the peak fraction number for the protein

Press A

The calculator will then display the elution volume of that protein

Press B

The calculator will then display the value of $(-\log K_{av})^{\frac{1}{2}}$.

APPENDIX IV

MOLECULAR WEIGHT AND FRICTIONAL RATIO DETERMINATIONS

This program, suitable for a Texas TI58/59 calculator, calculates the molecular weight and frictional ratio of any protein when supplied with the sedimentation coefficient and Stokes radius of that protein. The program uses the equations

$$M = \frac{6\pi\eta N a s}{(1-\bar{v}\rho)}$$

and $$f/f_o = a \left(\frac{3\bar{v}M}{4\pi N}\right)^{1/3}$$

Key in the following program:

Location	Key Code	Key
0		LRN
1	81	RST
2	91	R/S
3	76	2nd |b|
4	11	A
5	42	STO
6	05	05
7	95	=
8	65	×
9	43	RCl
10	01	01
11	55	÷

Location	Key Code	Key
12	43	RCl
13	02	02
14	95	=
15	42	STO
16	06	06
17	91	R/S
18	76	2nd |b|
19	12	B
20	43	RCl
21	05	05
22	55	÷
23	53	(
24	43	RCl
25	03	03
26	65	×
27	43	RCl
28	06	06
29	55	÷
30	43	RCl
31	04	04
32	54	)
33	22	INV
34	45	y^x
35	03	3
36	95	=
37	91	R/S
38		LRN

The following data must be entered into the memories:

1. The value of $6\pi N$

   Enter 1.1347433 EE 25 STO 01

2. The value of $(1-\bar{v}\rho)$

   Enter 0.275 STO 02

3. The value of $3\bar{v}$

   Enter 2.175 EE +/- 6 STO 03

4. The value of $4\pi N$

   Enter 7.5649551 EE 24 STO 04

Procedure:

Enter the sedimentation coefficient of the protein, e.g. if 7.7 S then enter 7.7 EE +/- 13

Press × (Key Code 65)

Enter the Stokes radius of the protein, e.g. if 6.4 nm then enter 6.4 EE +/- 9

Press A

The calculator will then display the molecular weight of the protein

Press B

The calculator will then display the frictional ratio of the protein.

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